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The mechanics and regulation of rat aortic smooth muscle contraction: implications of cytoskeletal remodeling, protein phosphorylations, and microtubule-based kinase transport

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The Mechanics and Regulation of Rat Aortic Smooth Muscle Contraction: Implications of Cytoskeletal Remodeling, Protein Phosphorylations, and Microtubule-Based Kinase Transport.

DISSERTATION

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To my advisor, Dr. Gary Wright, who taught me not only to do science, but also to think like a scientist, and who was a great man to work with...

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Thank you all for making this possible.

Abstract

The exact nature of the mechanisms and the regulation of vascular smooth muscle contraction is not well understood. To better understand these processes, we examined two systems involved in smooth muscle contraction, the cytoskeleton and the protein kinases. In order to study the role of the cytoskeleton in smooth muscle contraction, we examined the contractile and mechanical effects of cytoskeletal disruption. We found that the relationship between passive tension applied to aortic rings and the resulting increase in tissue length was nearly linear over the range of 1 g to 15 g. However, even with increasing tissue length, within the range of 1 g to 10 g passive tension, the total active force generated upon stimulation was not significantly changed. These observations emphasize the great flexibility of the mechanism(s) underlying the contractile response of vascular smooth muscle with regard to changes in tissue preload and length. Neither the blockade of microtubule polymerization by colchicine nor actin polymerization by cytochalasin B significantly changed the slope of the tissue length-passive tension preload curve indicating no effect on the tissues' capacity to stretch at a given preload. With stimulation of the tissue at different levels of stretch, colchicine caused an increase in the initial fast component of active tension development, but partially blocked the secondary slow rise in tension. Cytochalasin B dramatically reduced the total contractile response at each preload studied, and this effect was confined almost exclusively to the secondary slow increase in tension. When tissues were cooled to cause complete dissolution of the microtubule network and then warmed in the presence of

colchicine to prevent repolymerization of both the active and stable populations of microtubules, there was also a significant reduction in the slow component of contraction with no effect on the fast response. The partial blockade of synthesis of the microtubule-associated motor protein kinesin by application of an antisense oligonucleotide to aortae *in situ* or to aortic rings in tissue culture significantly reduced the contractile response to potassium depolarization. These results suggest that the microtubules and the actin filaments of the cytoskeleton play an active role in slow force development as opposed to a solely passive role based on the effect of the static, structural properties of these filaments on mechanical resistance. We propose that a tension-bearing element of the actin-containing cytoskeleton undergoes remodeling to adjust tension within the system. The microtubules could act through either the direct action of kinesin-mediated intracytoskeletal interactions in force development that involve a remodeling of the tension-bearing elements of the cytoskeleton or through the directed movement of the molecules involved in the transduction process.

Because the cytoskeleton and the protein kinases of smooth muscle are intimately linked, we examined the potential role of protein kinases in vascular smooth muscle contraction. We began by assessing the effects of a panel of specific kinase inhibitors on smooth muscle contraction. We found reductions in contraction with inhibition of myosin light chain kinase (MLCK), calcium-dependent calmodulin kinase (CaMKII), mitogen activated protein (MAP) kinase, and protein kinase C (PKC). Protein kinase C (PKC) is

translocated in an isoform-specific manner to distinct subcellular locations after stimulation of cells. It is thought that translocation is essential for PKC activation and that cellular localization underlies the PKC isoform-specific phosphorylation of substrate in the intact cell that is largely absent in *in vitro* assays. In the present studies, it was shown using Western blot analysis that the ratio of particulate to cytosolic PKC-α was reduced in rat aortic segments treated with colchicine to disrupt microtubular structure prior to stimulation with phorbol 12, 13 dibutyrate (PDB). Subsequent studies using laser confocal microscopy revealed that within thirty seconds after stimulation with PDB, PKC-α in cultured rat aortic smooth muscle cells changed from a diffuse cytoplasmic distribution to a highly structured filamentous pattern of staining. Dual immunostaining further indicated that the stimulation-induced filamentous pattern was due to co-localization of PKC-α with cell microtubules. At longer time intervals after PDB stimulation, PKC-α was observed to translocate to the perinuclear region of the cell. Disruption of the microtubular but not the actin-containing component of the cytoskeleton blocked the translocation of PKC-α to the perinuclear membrane. It was further shown that slow tension development, which has been reported to be selectively blocked by PKC antagonists in vascular smooth muscle, was also blocked by disruption of the cell microtubules. The results provide further evidence for the involvement of PKC in slow tension development by smooth muscle and indicate that PKC translocation may involve microtubular transport.

Table of Contents

List of Figures

I Literature Review

Smooth muscle was named due to its appearance when observed under early light microscopes. The nature of the smooth muscle cell was one of a homogenous base substance contained within a fusiform cell with a centrally placed nucleus. Upon further exploration, smooth muscle was found to be present in the cylindrical or hollow organs of six different systems: gastrointestinal, ocular, reproductive, respiratory, urinary and vascular. In each of these instances, with the exception of the ciliary muscle of the eye, the function of the smooth muscle is storage and transport, and because of this, smooth muscle is often considered a tissue of conductance and capacitance. This is not to say, however, that smooth muscle is an inert tissue, serving only as a conduit for various fluids. Within the gastrointestinal system, the smooth muscle of the stomach and intestines provides the motile force behind the directed movements of digestion. This function is mirrored in the reproductive and urinary systems. It is perhaps within the cardiovascular system, however, that the active nature of smooth muscle may best be observed. While the pumping of the heart is responsible for the propulsion of blood through the vasculature, it is the resting tone of the vessels that is the major determinant of the fluid dynamics of the system. This high degree of regulation within the cardiovascular system, as well as the functions carried out by the smooth muscle of other systems, is all due to the excitable nature of smooth muscle. In other words, smooth muscle has the ability to contract.

Following the discovery that smooth muscle could contract, the next questions were: in response to what, and by what mechanism. A convenient answer to the latter question was provided by data collected from skeletal muscle. Skeletal muscle, which falls under the classification of striated muscle, is characterized by its ordered arrangement of regularly spaced sarcomeres that move closer together upon contractile stimulation. Further investigation revealed the sarcomeres to be comprised of interdigitating filaments of actin and myosin. This finding was the basis for the sliding filament theory of muscle contraction first proposed by A.F. Huxley in 1957 and further elaborated on by H.E. Huxley in 1969. The basic principles of sliding filament theory are that the initial increases in intracellular calcium, found upon muscle stimulation, causes calcium to bind to troponin. This binding causes a shift of tropomyosin out of its resting position in the grooves within the actin helices, and this, in turn, causes a disinhibition of the interaction of actin and myosin. When inhibition is lifted, the binding of actin and myosin activates the ATPase in the myosin head, and stable crossbridges between the filaments are formed. Following this, the hydrolysis of ATP and the conformational change of myosin induced by hydrolysis provide the force necessary to pull the actin and myosin past each other. This repeats countless times in a ratcheting fashion. These biochemical events combined with the opposing interdigitation of the actin and myosin produce a shortening of each individual contractile unit, or sarcomere. In addition, because the sarcomeres are arranged in register, and because of the fixed nature of the muscle attachments, the simultaneous actin:myosin interactions in the sarcomeres produce a net shortening, or contraction, of the muscle as a whole.

Despite the presence of gross morphological differences between smooth and striated muscle, much of the initial work concerning the biochemistry and biophysics of smooth muscle contraction was analyzed and presented within the existing parameters that had been determined for striated muscle contraction. This basic framework became accepted as a viable model for the mechanisms of muscle contraction, regardless of cell type (Marston and Smith, 1985).

Contradictions to Convention in Smooth Muscle It is widely held that the principal biochemical and biophysical events underlying the contraction of smooth muscle are similar to those of skeletal and cardiac muscle. Stimulation of smooth muscle results in an elevation in intracellular calcium which binds to calmodulin. The calcium-calmodulin complex then activates myosin light chain kinase (MLCK) with a resulting phosphorylation of the Ser19 residue on the myosin light chains. This phosphorylation allows for activation of the myosin ATPase, cycling of myosin crossbridges along the actin filament, and the subsequent development of force. While there are these similarities in the initiation of contraction, there are a number of observations that bring the direct applicability of the sliding filament model to smooth muscle into question. Smooth muscle is capable of generating at least as much tension as striated muscle (Murphy, 1976). In striated muscle, the active force developed is in direct proportion to the degree of overlap of the actin and myosin filaments (Gordon et al., 1966). In smooth muscle, however, the association of myosin with actin filaments appears to be less well organized (Rice et al., 1970; Somlyo, 1980). Where a clear actin-myosin relationship is

observed, the actin filaments are arranged in bundles or rosettes of up to 15 filaments to each myosin. Moreover, the myosin content of smooth muscle may be as little as 20% of that of striated muscle (Murphy et al., 1974; Murphy, 1976). Given the arrangement of the myofilaments and the low contractile protein content, it is difficult to envision the mechanical or biochemical advantage which allows for the level of force developed by smooth muscle operating with only a sliding filament-based mechanism.

Smooth muscle has the unique ability to slowly develop force, and to then maintain this tension for extended periods (Figure 1). Furthermore, this maintenance of force occurs at levels of energy consumption which are about 0.35% of those seen in striated muscle (Paul, 1983). There is evidence to indicate that the slow force development and maintained tension (Driska et al., 1981) and velocity of shortening (Merkel et al., 1990) are dissociated from myosin light chain phosphorylation and actin-activated myosin ATPase activity (Zhang and Moreland, 1994) in smooth muscle. Most interestingly, it has been demonstrated that the phorbol ester- induced contraction of smooth muscle can occur in the absence of an increase in intracellular Ca^{2+} (Nakajima et al., 1993) or concomitant myosin light chain phosphorylation (Singer and Baker, 1987). Hence, there is a need to understand the differences in the biochemical mechanisms between striated and smooth muscle contraction.

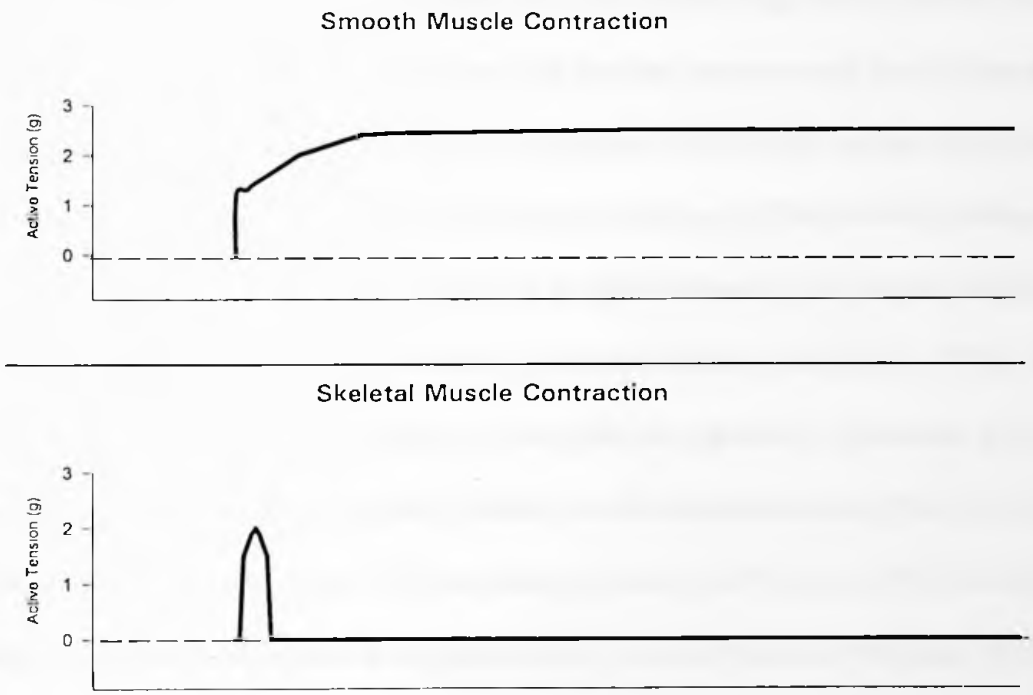

Figure 1 Graphic representation of the time course of tension development in smooth and striated muscles in response to a single activating stimulus. Smooth muscle is characterized by a rapid increase in tension followed by a slow generation of tension and maintenance of developed force. Striated muscle also rapidly develops active tension, but contraction quickly returns to baseline, with no maintenance of developed force.

Compensation for Low Myosin Levels in Smooth Muscle The myosin of smooth muscle is myosin II, similar to the myosin of striated muscle. Myosin II has two heavy chains with globular heads and α-helical tails. Covalently bound to the tails of the heavy chains are two types of light chains, the essential (ELC) and the regulatory (RLC) light chains. The globular heads of the heavy chains are mainly responsible for the work of filament sliding as they contain both the actin binding sequence and the ATPase activity. In vertebrate striated muscle, the light chains appear to have little impact on contraction as removal of both classes of light chains had no effect on ATPase activity (Wagner and Giniger, 1981). It has been speculated that, in striated muscle, the purpose of the light chains is to stabilize the myosin molecule (Sivaramakrishnan and Burke, 1982). In contrast, smooth muscle light chains, in particular the regulatory light chain, play a large role in contraction. Phosphorylation of Ser19 in the N-terminal end of the RLC is the essential switch that activates ATPase activity (Lowey and Trybus, 1995) and allows for actin binding and subsequent force generation. Even with these differences, the same basic mechanism of contraction is assumed to be at work. However, with such a low density of myosin, alternative theories were needed to explain the strong contractions developed by smooth muscle.

Many of the possible explanations offered to this problem have centered on the mechanical properties of the myosin molecule itself. Recent work has indicated that smooth muscle myosin translocates actin filaments at velocities as much as ten-fold slower than does striated muscle myosin (Lowey and Trybus, 1995). This could possibly

explain the generation of a slowly developing contraction. However, even with the wide range of velocities reported for smooth muscle myosins (Umemoto and Sellers, 1990; Warshaw et al., 1990; Trybus et al., 1994; Kelley et al., 1993), it cannot explain the rapidly developing fast component of the contraction which develops force with a velocity comparable to that of striated muscle, nor can it explain the high level of force generated by smooth muscle fibers.

Another possibility is that smooth muscle myosin may compensate for low density by increasing the force generated by each actin:myosin crossbridge. This could be achieved by an increase in the "duty cycle" – defined as the fraction of time that myosin remains attached to the actin filament during the hydrolysis cycle of ATP (Uyeda et al., 1990) – or an increase in the unitary force generated by smooth muscle myosin. Initial studies by Harris and Warshaw (1993) found that there were no differences between the duty cycles of smooth and skeletal muscle myosins; however, these studies were performed under "no-load" conditions. Under loaded conditions, smooth muscle myosin was found to be capable of generating crossbridge forces of three to four times those produced by skeletal muscle myosin (VanBuren et al., 1994; VanBuren et al., 1995). However, it is uncertain whether this is due to an increase in smooth muscle myosin duty cycle, increases in unitary force, or a combination of the two (Sellers, 1996).

A final possibility hinges upon differences in the packing of myosin monomers into filaments and the geometric arrangement of these filaments. While skeletal muscle

myosin monomers form bipolar filaments with a characteristic central bare zone, smooth muscle myosin aggregates into filaments in a "side-polar" orientation, leaving either end of the filament bare of myosin heads (Craig and Megerman, 1977; Cooke et al., 1989). In addition to this unique orientation of myosin monomers within the filament, the thick filaments of smooth muscle are also relatively longer than those of skeletal muscle (Ashton et al., 1975). These features would allow a large number of crossbridge interactions per myosin thick filament, thus increasing the force capable of being sustained by the filament (Hellstrand and Nordstrom, 1993).

Explanations for Low-Calcium Contraction Early work with permeabilized smooth muscle fibers, tissues in which the cellular membranes are "skinned" away from the cell thus allowing precise control of intracellular conditions, first indicated a dependence on calcium for contraction (Filo et al., 1965). However, this relationship has since been proven to not be correlative. For example, different agonists can produce similar degrees of force development with calcium levels held at different levels. Conversely, different agonists induce different degrees of force with equivalent changes in intracellular calcium (Kamm and Grange, 1996). In addition, contractions elicited by activation of protein kinase C may occur in the absence of a rise in intracellular calcium levels (Nakajima et al., 1993). These findings have given rise to a number of theories regarding the calcium sensitivity of smooth muscle contraction and the regulation of this property.

The calcium sensitivity of smooth muscle, defined as the degree of contractile activation

elicited in response to a given concentration of intracellular calcium, can be either up-regulated or down-regulated. Activation of myosin light chain kinase is accomplished by association with the calcium-calmodulin complex (Ca:CaM). If, however, the myosin light chain kinase is itself phosphorylated, the sensitivity of the kinase to activation by Ca:CaM is decreased, thus requiring higher levels of intracellular calcium to achieve contraction (Conti and Adelstein, 1981). This phosphorylation occurs near the Ca:CaM binding site (Tansey et al., 1994), and can be accomplished *in vitro* by protein kinase C or by calcium-calmodulin dependent protein kinase II (CaMK II) (Kamm and Grange, 1996). However, in intact tissues, activation of protein kinase C has little to no effect on the phosphorylation of myosin light chain kinase, suggesting that *in vivo*, it is CaMK II that is responsible for desensitization to calcium (Stull et al., 1990; Van Riper et al., 1995). While an understanding of this system may help to explain differences in degree of contraction at equal levels of intracellular calcium, it does not explain how smooth muscle can contract with low or no rises in intracellular calcium levels. An explanation for this comes from work done with myosin light chain phosphatase.

Very little is known about the action and regulation of myosin light chain phosphatase (MLCP); however, recent work suggests that activation of MLCP allows equal degrees of smooth muscle contraction at lesser levels of intracellular calcium (Kamm and Grange, 1996). In smooth muscle, the amount of calcium necessary for half maximal myosin light chain activation is much higher for tissues depolarized with potassium than for tissues stimulated with agonists (Rembold and Murphy, 1988; Karaki et al., 1989; Tang et al.,

1992). This effect can also be elicited by the application of GTP to the preparation (Nishimura et al., 1988; Kitazawa et al., 1989), and this GTP effect is associated with increased phosphorylation of myosin light chain (Kubota et al., 1992). It appears that this increase in phosphorylation is not due to a potentiation of myosin light chain kinase activity by the GTP, but rather to an inhibition of myosin light chain phosphatase activity (Kitazawa et al., 1991). It is believed that this effect of GTP is reliant upon guanine nucleotide binding proteins, and, in fact, the GTP-binding proteins *rho*A p21 and *ras* p21 can cause increases in calcium sensitivity (Hirata et al., 1992). While evidence points to the possibility that agonist binding to G-proteins may elicit an inhibition of myosin light chain phosphatase activity, the precise second messenger system by which this occurs has yet to be revealed.

Other possible candidates for regulating calcium sensitivity in smooth muscle are the calcium binding proteins caldesmon and calponin. Both caldesmon and calponin can be phosphorylated by CaMK II or PKC, and calponin phosphorylation reverses the inhibition of myosin ATPase imparted by caldesmon (Winder and Walsh, 1993). These proteins appear to be complementary to one another, although they seem to be localized to different actin filament sub-populations (North et al., 1994) Several findings have pointed to caldesmon as a potential regulator of calcium sensitivity. *In vitro* preparations of actin filaments and caldesmon show an increased sensitivity to calcium that directly correlates with the amount of caldesmon present (Marston and Smith, 1984). In addition, in a similar preparation, addition of anti-caldesmon antibodies negated this enhanced

sensitivity (Marston et al., 1988).

Possible Mechanisms for High-Economy of Contraction The first suggestion that smooth muscle was unique in its energy utilization was the finding in 1930 that contracted smooth muscle could maintain developed force with very low energy output (Bozler, 1930). Bozler also showed that although force was maintained at a constant level, heat production decreased with time, indicating an increase in economy of contraction as the contraction progressed. This finding is reasonable when the biological function of the tissues comprising smooth muscle is taken into consideration. In later work, Bozler went on to postulate that this high economy of smooth muscle contraction was due to a reduction in the rate of detachment of myosin from actin during crossbridge cycling (Bozler, 1977), but again, no mechanism was proposed for this slowing of the crossbridge cycle. A possible explanation came with the finding that while myosin light chain phosphorylation was necessary for smooth muscle contraction (Kamm and Stull, 1985; Hartshorne et al., 1989), high force could be generated and maintained with very low levels of myosin light chain phosphorylation (Moreland and Moreland, 1987; Ratz and Murphy, 1987). These works were and are still the foundation for current theories of crossbridge cycling, most notably the latch hypothesis.

The latch hypothesis, proposed by Dillon et al. in 1981 and characterized further by Hai and Murphy (1988,1989) and Murphy (1989), describes a "latch" state in which dephosphorylated myosin cross-bridges remain attached to actin but cycle slowly to

produce slow increases in tension or maintenance of tension at low levels of myosin light chain phosphorylation. This theory is based on the premise that there are 4 possible states for myosin to assume: (M), unattached myosin; (AM), myosin attached to actin; (Mp), unattached myosin which has been phosphorylated on the light chain; and (AMp), attached and phosphorylated myosin (Hai et al., 1991). According to this theory, an initial phosphorylation of the myosin regulatory light chain initiates the contraction in the presence of increased intracellular calcium. This period of high MLC phosphorylation is responsible for the rapid initial shortening velocity of smooth muscle tissue. As calcium levels decline, myosin light chain kinase activity declines concomitantly; however, force continues to develop because there is a transition from (AMp) to (AM). This continued attachment of dephosphorylated myosin allows for a slower detachment constant from actin and for maintenance of force by these crossbridges. Further, the small levels of myosin that remain in the (AMp) state can continue to develop force, thus accounting for the slower shortening velocity for smooth muscle contraction. Many mathematical models have been produced using rate constants believed to be consistent with latch, and these models accurately predict many parameters of smooth muscle contraction (Walker et al., 1994); however, there is little direct experimental evidence for latch theory.

It has been further suggested that secondary mechanisms regulate the rate of high-affinity cross-bridge cycling, or that mechanisms independent of myosin light chain phosphorylation determine slow tension development in smooth muscle. Somlyo et.al. (1989) proposed that slow force development is due to the balance of activity between

MLCK and protein phosphatase in the phosphorylation/ dephosphorylation of myosin light chain. The subsequent identification of a specific myosin light chain phosphatase (Shirazi et al., 1994; Somlyo and Somlyo, 1994), provided further evidence for this hypothesis. Somlyo's hypothesis predicts that inhibition of myosin light chain phosphatase would cause an increase myosin light chain phosphorylation, increase myosin ATPase activity, and increase the rate of tension development. However, it was found that inhibition of protein phosphatase activity, which according to this theory would alter the relationship between force and MLC phosphorylation, had no significant effects on this relationship (Siegman et al., 1989). This would imply that the high force developed in the presence of only low levels of myosin light chain phosphorylation must rely on a process other than dephosphorylation of the myosin crossbridges. (Siegman et al., 1991). This discovery led to the suggestion of yet another means of crossbridge regulation, namely, cooperativity. In this hypothesis, either low degrees of myosin light chain phosphorylation turn on all the crossbridges for force production or only a few select crossbridges are ever phosphorylated to generate force (Vyas et al., 1992). It is uncertain how this process is regulated; however, both the actin filament itself and its associated proteins have been proposed to play a part.

Several specific regulatory proteins have been suggested that may initiate or enable the latch state. One of these is kinase related protein (KRP) (Collinge et al., 1992; Shattuck et al., 1989) which has also been referred to as telokin (Ito et al.,1989; Gallagher and Herring, 1991). Normally, in *in vitro* studies, unphosphorylated myosin dissociates into

its monomeric form upon addition of MgATP (Craig et al., 1983; Trybus et al., 1982). However, in intact smooth muscle preparations, filamentous myosin remains intact even at myosin light chain phosphorylation levels of less than five percent (Somlyo et al., 1981; Gillis, et al, 1988), clearly indicating that other factors must be present *in vivo* that stabilize unphosphorylated myosin. One of these factors is postulated to be KRP (Shirinsky et al., 1993). The gene for KRP is found within the gene for myosin light chain kinase (Collinge et al, 1992; Shoemaker et al., 1990) and is thought to be very abundant in smooth muscle (Shirinsky et al., 1993). This protein, *in vitro*, binds to unphosphorylated myosin and reverses the depolymerizing effect of MgATP, thus allowing for retention of thick filament integrity even in an unphosphorylated state (Shirinsky et al., 1993).

Another protein proposed to play a regulatory role in actin:myosin crossbridge cycling is the actin-binding protein caldesmon. Caldesmon has a high binding affinity for actin (Drabrowska and Galazkiewicz, 1986), and this affinity is sensitive to changes in intracellular calcium levels. In addition, caldesmon interacts with myosin (Ikebe and Reardon, 1988) and has been shown to inhibit both actin:myosin binding (Nowak et al., 1989) and actin-activated myosin ATPase activity (Hemric et al., 1988; Lash et al., 1986). Caldesmon inhibitory activity is reversed by Ca^{2+} and calmodulin (Lash et al.,1986) or by its phosphorylation (Ngai et al., 1987). Because the phosphorylation of caldesmon is markedly increased during maintained tension, it has been suggested that the phosphorylation state of this protein functions as an on/off switch for the latch-state

cross-bridge cycling (Adam et al., 1989).

Additionally, two other thin filament-associated proteins, calponin and caltropin, have been purported to play a role in crossbridge regulation. Calponin binds to both actin and to calmodulin, and inhibits myosin ATPase activity *in vitro*, and is distributed along the actin filaments in a manner similar to that of troponin C in skeletal muscle (Takahashi et al., 1988). Because it is the troponin-tropomodulin system that imparts cooperativity to the crossbridge systems of skeletal muscle, a role for calponin in crossbridge regulation was examined. It was found that the presence of calponin in *in vitro* systems increased the amount of generated force while causing a decrease in myosin ATPase activity. From this it was suggested that calponin may stabilize dephosphorylated crossbridges when the myosin was in the (AM) state (Haeberle, 1994). The calponin induced inhibition of ATPase activity is reversed by phosphorylation of the protein which decreases its affinity for actin (Winder and Walsh, 1989). However, *in vivo* studies do not show significant phosphorylation of calponin. As an alternative to phosphorylation, several studies have suggested that caltropin may mediate the binding of calponin to actin (Willis et al., 1994; Mani and Kay, 1993), although the nature of this mechanism has not been elucidated.

While all of these hypotheses focus on the properties of the actin:myosin crossbridge, another area of work has focused on the role of the non-actin:myosin cytoskeleton in explaining smooth muscle contraction. A significant development in this direction was the analysis of the potassium-induced contraction of aortic smooth muscle on the basis of

its initial fast rise in tension followed by a slow, strong development of tension and maintenance of this tension (Wright and Hurn, 1994). The fast phase of the contraction has distinct similarities to the contraction of striated muscle contraction in that it occurs in the presence of high intracellular calcium concentrations and high levels of myosin light chain phosphorylation. Additionally, this portion of the contraction displays a length-force relationship similar to that of skeletal muscle. In contrast to the fast component, in the slow component of the smooth muscle contraction, which accounts for approximately sixty percent of the total force generated, active tension is developed in the face of diminishing intracellular calcium and minimal myosin light chain phosphorylation. Also, the length-force relationship for this part of the contraction is markedly different from that of either the fast phase of smooth muscle contraction or of skeletal muscle contraction. These observations led to the suggestion that different mechanisms contributed to the fast and slow components of smooth muscle contraction.

Rasmussen, et al. (1987) first proposed that the initial fast and secondary slow phases of the contractile response are mediated by different mechanisms. This hypothesis incorporates the observation that the actin cytoskeleton consists of two domains: a continuous longitudinal arrangement of actin and myosin filaments with associated regulatory proteins, and an arrangement of actin and intermediate filaments which are attached to cell surface plaques and linked to intracellular dense bodies (Small, et al., 1986; Cooke and Fay, 1972). It is proposed that the mechanism underlying the fast phase centers on the interaction of actin and myosin; whereas, the secondary slow response

involves a phosphorylation-mediated rearrangement of filaments to lengthen or shorten the system via linkages between filamin and actin filaments at the dense bodies, presumably effecting changes in the mechanical advantage within the system. Most recently, Pratusevich, et al. (1995) have proposed that the long working range of smooth muscle could be explained by a plastic rearrangement of myosin resulting in more myosin thick filaments in series at longer lengths. They showed that tetanic force was below its steady state after either tissue stretch or release but increased to a steady level within 5 or 6 tetanic contractions. They suggest that this phenomenon was due to temporary force depression due to length change followed by reforming of the filament lattice to produce optimal force development.

The Cytoskeleton The smooth muscle cytoskeleton is comprised primarily of three basic components: actin microfilaments, microtubules and intermediate filaments. These three elements are the building blocks for the various structures present within the smooth muscle cell, however, a much larger body of work exists on the location and function of the actin and the intermediate filaments.

The actin cytoskeleton is involved in vesicle trafficking, cell division, muscle contraction and cell motility (Barkalow and Hartwig, 1995) and can exist as either monomeric, G-actin, or as filamentous, F-actin. The assembly of G-actin into F-actin is a two-step process. The first step, nucleation, is the rate limiting step in polymerization and consists of the association of three actin monomers into a nucleus. Once this nucleus is formed,

rapid elongation of the filament occurs (Korn, 1982). The assembling actin filament displays a polarity in its construction, this polarity may be demonstrated by decoration of the actin filament with myosin. The myosin binds the actin filament at an angle, thus producing what are commonly referred to as the barbed and pointed ends of the actin filament. The barbed end of the actin filament has a higher affinity for monomeric actin and therefore is kinetically favored for assembly.

The actin found in smooth muscle is structurally similar to the actin found in most cells and has comparable molecular weight, ability to polymerize, to attach to myosin and to activate myosin ATPase activity (Bray,1972). There is also a high degree of conservation of actin sequence within cell types with a 95 percent amino acid sequence homology (Pollard and Cooper 1986). In vertebrate tissues, there are six distinct actin isoforms, α-skeletal, α-cardiac, α-vascular, γ-enteric, γ-cytoplasmic and β-cytoplasmic. Each of these is encoded by a distinct gene (Vandekerckhove and Weber, 1978; Reddy et al., 1990), and three isoforms are present in smooth muscle. The isoforms found in smooth muscle are α-actin, β-actin, and γ-actin (Herman, 1993) and differ only in their N-terminus (Vandekerckhove and Weber, 1981). These isoforms are categorized into muscle (α and γ- actin) and cytoplasmic (β and γ- actin) divisions. Of the cytoplasmic actins, β-actin is the most predominant, comprising up to thirty percent of the total cellular actin complement (North et al., 1994). Within the smooth muscle cell itself, there appears to be a functional compartmentalization of these isoforms. Antibody staining against β-actin produces diffuse staining throughout the cytoplasmic

compartment, whereas antibodies directed toward the α and γ-actin labels the myosin-containing contractile region (North et al., 1994). Small (1986) suggested that this compartmentalization was a reflection of function. He hypothesized that contraction was generated through the actin:myosin containing portion and resting muscle tone was provided by the cytoplasmic actin containing portion of the cell. It has also been proposed that the underlying mechanism for the development and maintenance of slow smooth muscle contraction lies not in the actin:myosin portion of the actin cytoskeleton, but in the β-actin containing, or cytoskeletal, fraction of the smooth muscle actin cytoskeleton (Wright and Hurn, 1994; Battistella-Patterson et al., 1997). This theory was precipitated by the discovery that inhibition of actin polymerization could selectively and reversibly inhibit the slow phase of potassium-induced contraction (Wright and Hurn, 1994).

Inhibition of actin polymerization is accomplished by the application of cytochalasins to the smooth muscle tissue. Cytochalasins, from the Greek *cyto*, cell and *chalasis*, relaxation, are a class of fungal metabolites first derived from cultures of *Helminthosporium dematioideum* and *Metarrhizium anisopliae* in the late 1960's (Carter, 1967). These compounds were initially described to prevent cytoplasmic cleavage during cell division, inhibit cell motility and restrict membrane ruffling. All these activities now can be attributed to the actions of actin polymerization, however, at the time the loss of these functions was explained as an effect of the cytochalasins on the cell to glass interface of the cultured cells utilized (Carter, 1967). Later the cytochalasins were shown

to cause microfilaments to lose their filamentous nature (Schroeder, 1970; Wessells et al., 1971), and these microfilaments were then shown to be, in fact, actin filaments (Spudich and Lin, 1972; Spudich, 1972). Cytochalasin inhibits actin polymerization by binding to the barbed end of actin filaments, inhibiting any addition of actin monomer to the existing filament (Cooper, 1987).

It was clearly demonstrated that smooth muscle contraction could be inhibited by treatment with cytochalasin, however, it was unknown if this was through the effects of cytochalasin on the polymerizing actin cytoskeleton or due to some other effect of the drug (Wessells, et al, 1971). Because stable actin, such as is found in striated muscle, is impervious to depolymerization by cytochalasin, and because the cytoskeletal actin compartment was not believed to play a role in smooth muscle contraction, it was suggested that the mechanism of action of cytochalasin on smooth muscle contraction was through the blockade of calcium influx (Dresel and Ogbaghebriel, 1988), blockade of glucose uptake (Dresel and Knickle, 1987), inhibition of myosin light chain phosphorylation or inhibition of myosin ATPase activity. Each of these possibilities has been addressed. Obara and Yabu (1994) showed that treatment of smooth muscle tissues with cytochalasin at a concentration that induced maximal inhibition of the contraction, had no effect on calcium currents, myosin light chain phosphorylation or myosin ATPase activity in response to high potassium contraction. This work was verified in carbachol treated tissues and in addition, the absence of an effect of cytochalasin on glucose transport at the concentration necessary to produce maximal inhibition was shown (Tseng

et al., 1997). At the same time that these possibilities were being refuted, it was suggested that it was the effect of cytochalasin on the actively polymerizing actin cytoskeleton that was eliciting inhibition of slow phase contraction (Wright and Hurn, 1994). This, in addition to the finding that skeletal and cardiac muscles are unaffected by cytochalasins has led to the proposal of a new theory (Battistella-Patterson et al., 1997). Because the stable actin of skeletal and cardiac muscle is not affected by cytochalasin, it was reasoned that the fast phase, which is similarly unaffected by cytochalasin, was produced by a mechanism comparable to sliding filament based systems. This hypothesis discusses the role of the dynamic actin cytoskeleton as a potential player in the development of slow smooth muscle contraction, however, the exact mechanisms by which this occurs have yet to be elucidated.

There are also several actin-binding proteins that also bind to calcium and that are believed to play a role in contractile function. The first of these, caldesmon has been discussed above in relation to its contributions to crossbridge regulation and to calcium sensitivity. The caldesmon of smooth muscle, when isolated, is found to be associated tightly with the actin filaments (Marston and Lehman, 1985), and when examined *in vivo* is found to be incorporated into the actin filaments of the contractile domain (Furst et al., 1986; North et al, 1994) in an actin to caldesmon ratio of sixteen to one (Marston, 1990; Lehman et al., 1993). The caldesmon molecule is a long, rod-like structure (Mabuchi and Wang, 1991), and nuclear magnetic resonance (NMR) analysis indicates that it is composed of several rod sections linked by flexible hinge regions (Stafford et al., 1990).

The caldesmon molecules are oriented in an extended form along the actin filament and beside tropomodulin. This organization provides the basis for the function of caldesmon in contraction (Marston and Huber, 1996). Several groups have shown, *in vitro*, that caldesmon affects actin motility (Shirinsky et al., 1992; Haeberle et al., 1992; Okagaki et al., 1991), and more recent work has proposed that this inhibition is due to a switching off of actin filaments in units as opposed to affecting actin:myosin interactions on a single filament basis (Fraser and Marston, 1995). This has led to the proposal that caldesmon, in addition to its effects on calcium sensitivity and crossbridge dynamics, may act as troponin does in skeletal muscle to regulate actin movement (Marston and Huber, 1996).

In addition to the actin cytoskeleton, there is another portion of the smooth muscle cell that has been closely examined as playing a role in the contractile process. The intermediate filaments are a diverse group of proteins that play many roles within the cell. Upon electron microscopic analysis, there appear many electron-dense regions, these are referred to as either membrane associated dense bodies (MADBs) or as cytoplasmic dense bodies (CDBs), and are both comprised primarily of intermediate filaments (Stromer, 1995). The MADBs are, as their name implies, found at the cell periphery and have been shown to contain α-actinin (Fay et al., 1983; Geiger et al., 1985), vinculin (Geiger et al., 1985; Volberg et al., 1986), metavinculin (Belkin et al., 1988), talin (Volberg, et al, 1986), filamin (Small et al., 1986), plectin (Wiche et al., 1983) and actin. CDBs are found in the interior of the cell and contain α-actinin (Fay et al., 1983; Small et al, 1986), desmin (Berner et al., 1981), filamin (Lehman et al., 1987) and vimentin (Schmid et al.,

1982). Both of these structures are believed to anchor actin filaments, and it is possible that they are important to smooth muscle contraction. Both desmin (Inagaki et al., 1988) and vimentin (Inagaki et al., 1987) can be phosphorylated by protein kinase C (PKC), and this phosphorylation is related to a disassembly of the filaments. Additionally, it has been shown that smooth muscle desmin is phosphorylated in response to agonist simulation of contraction (Park and Rasmussen, 1986), and that this phosphorylation is reversible upon relaxation of the tissue (Barany et al., 1992). This reversible phosphorylation supports the theory that there is a domain of smooth muscle comprised of filamin, actin and desmin (FAD), which is phosphorylated during smooth muscle contraction (Rasmussen et al., 1987). This phosphorylation would allow partial depolymerization of the filaments and a rearrangement of the cytoskeleton to maintain force with low energy expenditure.

While both the role of the actin cytoskeleton and the intermediate filaments in smooth muscle contraction have been widely studied, the role of the microtubules in smooth muscle physiology has largely been ignored. The microtubules are responsible for many different functions including formation of the mitotic spindle, axonal and dendritic extension in neurons, maintenance of intracellular organization, transport of various compounds and organelles (Avila, 1991) and cell motility (Bershadsky and Vasiliev, 1993). The microtubules exhibit the ability to extend and retract by polymerization and depolymerization, respectively. This polymerization or depolymerization is accomplished by the addition or removal of tubulin monomers (Sullivan, 1988). Like actin filaments, the microtubules have a distinct polarity. At the plus end the addition of

tubulin monomers occurs at a greater rate than at the minus end. There are three types of tubulin that comprise the microtubule, α, β and γ. The α and β-tubulin form dimers and these dimers then attach to the growing microtubule. γ-Tubulin is found at the centrosome and forms the nucleation site for further polymerization (Oakley and Oakley, 1989; Zheng et al., 1991). This site is often referred to as the microtubule organizing center (MTOC). The MTOC not only serves as a point of origin for all the cellular microtubules, it also maintains microtubular polarity throughout the cell because it constitutes the minus end of the microtubule. This degree of organization ensures that microtubular polymerization can only occur in a direction away from the MTOC (Brinkley, 1985). The importance of this polarity can be seen in the examination of two proteins associated with the microtubules. In order for the microtubules to fulfill many of the functions that they perform, there must be a means to move things along the microtubules in both directions. This is accomplished by microtubule associated motor proteins, notably kinesin and dynein. Both of these proteins consist of a heavy chain and of a light chain, and in both cases it is the heavy chain that binds to the microtubule and generates force through hydrolysis of ATP, while the light chain is responsible for binding a specific "cargo". A major difference between the two motor proteins is based on the polarity of transport along the microtubules; kinesin is a plus-end oriented motor, moving away from the MTOC, and dynein is a minus end-directed motor, moving toward the MTOC. This type of system allows for specific and directed bi-directional transport of compounds and organelles.

In the study of various functions of the microtubules in living systems, a method that has been widely employed has been the use of the drug colchicine, which causes depolymerization of the microtubules into free tubulin. In early studies on cultured skeletal muscle cells, colchicine was found to cause a disruption of the cellular morphology, producing "fractures" within the cytoplasm (Godman, 1955). In addition, the application of colchicine to these cells caused a loss of mitochondrial orientation, however, both of these effects were reversible with the removal of colchicine (Godman, 1955). At the time, the microtubules had not yet been discovered, but it had been theorized that colchicine "might effect a folding of elongated protein chains from fibrous to globular" (Ostergren, 1944), or that it acted by causing a "dissociation of micellar aggregates by directly disrupting their linkages" (Simms and Sanders, 1942). It is now known that colchicine works by binding to the polymerized tubulin which results in depolymerization of the microtubule.

Employment of *in vivo* Antisense Techniques The application of antisense oligonucleotides to block synthesis of specific proteins has become increasingly common. These synthetic oligonucleotides are complementary to a specific mRNA and by binding the mRNA prevent its translation to protein or targets the RNA duplex for degradation. This allows for a highly specific means of reducing the amounts of a single protein. Because oligonucleotides are hydrophilic, they do not readily pass through the cell membrane. In order to improve the efficiency of uptake, the oligonucleotides must either be modified or conjugated to another compound to increase their hydrophobicity and,

therefore, increase their membrane permeability. Many methods have been employed to accomplish this. One method involves a pre-incubation of the oligonucleotides with a cationic lipid solution. With gentle mixing and a short incubation period, the oligonucleotides become encapsulated within micelles of the cationic lipid which may readily penetrate the membrane (Schreier, 1994). Another method that has been used is the incorporation of the oligonucleotides into a detergent-like pluronic gel (Simons et al., 1992). In this case, the low hydrophobicity of the oligonucleotides is compensated for by the permeabilization of the membrane by the pluronic gel. This method is used primarily for *in vivo* applications because the consistency of the gel allows for localized application of the oligonucleotides. A third method utilizes a virus-mediated incorporation of sequence coding for RNA that is complementary to a specific message into the cells (Lee et al., 1996). This method is used in both *in vivo* and *in vitro* situations and may result in widespread delivery of antisense oligonucleotides to tissues and organs *in vivo*.

Kinases of Smooth Muscle Contraction There are numerous kinases that are abundant in smooth muscle, however, only a few have been distinguished as playing a role in the generation and regulation of contraction. The first of these, myosin light chain kinase (MLCK), is the principal kinase associated with smooth muscle contraction. The role of myosin light chain kinase in smooth muscle contraction has been discussed above. Briefly, upon agonist stimulation or depolarization of the smooth muscle tissue, there is a rise in intracellular calcium levels which precipitates the association of calcium with calmodulin. This complex then, in turn, binds to MLCK. Binding of calcium:calmodulin

to MLCK activates the kinase, which then is able to phosphorylate the myosin regulatory light chain. Light chain phosphorylation results in the initiation of crossbridge cycling and subsequent contraction. Myosin light chain kinase is a 130 kDa protein (Gallagher et al., 1991) that exists in two distinct forms: those found in skeletal muscle (Herring et al., 1992) and those found in smooth muscle (Gallagher et al., 1991; Kobayashi et al., 1992). It is present in much higher amounts in smooth muscle (Stull et al., 1986). The N-termini of these kinases contains the actin binding region of the protein (Kanoh et al., 1993) and is highly conserved within both muscle types and between species (Stull et al., 1996). The central region of each kinase is highly variable with considerable differences between species being common. The catalytic core immediately follows the central region and is not only highly homologous within myosin light chain kinase forms, but is also similar to the catalytic regions of several other kinases (Knighton et al., 1991; DeBondt et al., 1993; Zhang et al., 1994). The C-terminal region of the kinase is the regulatory domain, and within this portion of the kinase, there are two specific functions, autoinhibition and calmodulin binding. The autoinhibitory region of the regulatory domain is adjacent to the catalytic core and is folded over the catalytic region, blocking activity until calmodulin binding causes a conformational change that removes the inhibition (Kemp et al., 1994). Much of the work done to examine the function of MLCK has involved the use of inhibitors of the kinase, and many of these inhibitors are targeted to the autoinhibitory domain (Kemp et al., 1991). Early attempts to inhibit MLCK utilized its dependence upon calmodulin and acted to bind free calmodulin. These quickly lost their appeal when it was discovered that they were also inhibiting another kinase involved in smooth muscle

contraction, calcium/calmodulin dependent protein kinase (Asano and Stull, 1985; Nakanishi et al., 1992).

The calcium/calmodulin dependent protein kinase (CaMKII) family consists of four isoforms (α, β, γ and δ) regulated by the complex of calcium and calmodulin, and also by autophosphorylation (Hanson and Schulman, 1992). The kinase is responsible for many functions, including neurotransmitter release (Llinas et al., 1985), catecholamine synthesis (Waymire et al., 1988), mitosis (Baitinger et al., 1990), transcription (Sheng et al., 1991), vascular smooth muscle cell migration (Pauly et al., 1995) and smooth muscle contraction (Edelman et al., 1990; Tansey et al., 1992). It seems that there is a partitioning of isoforms with the α and β forms expressed almost exclusively in the brain and the γ and δ isoforms found in peripheral tissues (Bulleit et al., 1988; Tobimatsu and Fujisawa, 1989), with the δ isoform being predominant in smooth muscle (Schworer et al., 1993). Two of the possible effects of CaMKII on smooth muscle contraction have been discussed above. The first of these is an effect of phosphorylation by CaMKII on myosin light chain kinase. When MLCK is phosphorylated by CaMKII *in vivo* the affinity of MLCK for calmodulin is decreased and the calcium/calmodulin threshold for activation of MLCK is raised (Stull et al., 1990). In addition, if the CaMKII specific inhibitor, KN-62 is used in these preparations, the phosphorylation of myosin light chain by MLCK is potentiated, indicating that there is an inhibitory effect of CaMKII phosphorylation on MLCK activity (Tansey, et al., 1992). Calmodulin kinase II also has been shown to co-purify with caldesmon in smooth muscle preparations (Ngai and Walsh,

1985) and to phosphorylate caldesmon. This phosphorylation reverses the inhibitory effect of caldesmon on myosin ATPase activity (Ngai and Walsh, 1987). One problem with the theory that CaMKII is responsible for caldesmon phosphorylation during contraction is that the sites phosphorylated by CaMKII *in vitro* do not correspond to sites phosphorylated in intact tissues (Adam et al, 1989, 1992). The sites that are phosphorylated *in vivo* are the same as the sites phosphorylated by another kinase purported to be involved in smooth muscle contraction, mitogen activated protein (MAP) kinase (Adam et al, 1992). This suggests that if CaMKII is involved in regulating caldesmon phosphorylation and subsequently smooth muscle contraction, these effects may be due to the effects of CaMKII on MAP kinase (Singer et al, 1996).

Mitogen activated protein (MAP) kinase is a part of a family of serine/threonine kinases that are activated by growth stimuli. These kinases are present in almost all tissues and are activated only when phosphorylated on both tyrosine and threonine. This phosphorylation can be provided by the actions of a variety of agonists and stimuli including insulin (Clarke, 1994; White and Khan, 1994), insulin-like growth factor (Lamy et al, 1993), platelet derived growth factor (Chao et al., 1994), vasopressin, angiotensin II, growth hormone and muscarinic agonists (Alessindrini et al., 1992; Campbell et al., 1992; Yin and Yang, 1994). The actions of MAP kinase in smooth muscle are proposed to be via its phosphorylation of caldesmon (Adam et al., 1992). As discussed above, caldesmon inhibits myosin ATPase activity and its phosphorylation causes this inhibition to disappear. Additionally, this phosphorylation may alter the dynamics of actin

polymerization and organization in the smooth muscle cells (Adam, 1996). However, the pathway that activates MAP kinase in smooth muscle is not known. In many instances, MAP kinase activation is accomplished via membrane associated G-proteins that then transduce the signal to MAP kinase, but the exact signaling pathways involved are not yet entirely clear (Adam, 1996). One possible route of activation for MAP kinase is via another kinase, protein kinase C (Khalil and Morgan, 1993).

The protein kinase C family is a large, multifunctional group consisting of at least eleven isoforms divided into three subgroups based on their structure and activation requirements (reviewed by Ohno et al., 1991). The "conventional" PKCs (α, β, γ) require calcium, phosphatidyl serine, and diacylglycerol for full enzyme activity. The "novel" PKCs (ϵ, δ, θ, η) are calcium independent, and the "atypical" PKCs (ζ, ι, λ) are both calcium and diacylglycerol -independent but both require phospholipid, the only cofactor shared among all three groups. Each cell type has its own unique complement of PKC isoforms (Dekker and Parker, 1994). Additionally, within a given cell type, PKC may be involved in the regulation of a variety of diverse functions ranging from growth and differentiation (Nishizuka, 1992) to secretory activity (Kiley et al., 1992), motility (Bershadsky and Vasiliev, 1993), and signal transduction. The presence of multiple isoforms regulating diverse cellular functions suggests that each isoform phosphorylates a specific substrate and that redundancy is limited or absent (Mochly-Rosen, 1995). This, in turn, implies a high level of PKC isoform compartmentalization and regulatory control of activation.

The fundamental questions surrounding the regulation of PKC activity are: (1) the mechanism(s) underlying its movement to isoform-specific sites in the cell, (2) its activation, and (3) substrate specificity. Prior to cellular stimulation, PKC is found either diffusely throughout the cytoplasm or localized within particular regions of the cell (Mochly-Rosen, 1995). In order to be activated, PKC must be translocated to the cellular membranes. PKC translocation is both rapid and highly directional with detectable migration of the isoform observed within seconds after stimulation and peak movement achieved within an interval of minutes (Haller et al., 1990). The efficiency and membrane-directed nature of PKC translocation is thought to be due to a calcium-induced conformational change in the kinase which exposes hydrophobic regions within its structure that impart increased affinity for membrane binding sites (Bosca and Moran, 1993). There is now considerable evidence suggesting that the stable interaction of PKC with the membrane is achieved through binding to isoform-specific anchoring proteins termed receptors for activated C-kinase (RACKs) (Mochly-Rosen, 1995). Hence, it is thought that following stimulation-induced increases in intracellular calcium PKC migrates, presumably by diffusion, to membrane bound anchor proteins. The weak, specific interaction between the kinase and the anchor protein then serves to tether the kinase in close association with substrate achieving differential compartmentalization and isoform specificity of substrate phosphorylation.

The PKC isoforms found in vascular smooth muscle (α, β, ϵ, δ, ζ) (Andrea and Walsh, 1992) have been proposed to play a key role in the proliferation, differentiation

(Montesano and Orci, 1985), and contractile properties (Rasmussen et al., 1987) of these cells. The impact of PKC on contraction of vascular smooth muscle was indicated by the discovery that phorbol esters, analogues of diacylglycerol, caused a slowly developed but robust and sustained contraction (Danthaluri and Deth, 1984). Furthermore, PKC is tonically activated in response to a variety of contractile stimuli (Haller et al., 1990; Singer et al., 1992), while inhibition of PKC activity blocks or attenuates the contractile response to different agonists (Merkel et al., 1991; Shimamoto et al., 1993; Wright and Hurn, 1994). The down regulation of PKC protein by long-term exposure to phorbol ester has been demonstrated to attenuate the contractile response of smooth muscle to different agonists (Merkel et al., 1991). The change in the pattern of phosphoproteins during contraction is suggestive of a role for PKC in the slow phase of contraction unique to smooth muscle in which tension is slowly developed and may be maintained at a constant level for extended intervals (Haller et al., 1990). Other work has further shown that the application of staurosporine at a concentration ($8x10^{-9}M$), which should specifically inhibit PKC activity, caused the selective inhibition of slow tension development by rat aortic smooth muscle (Wright and Hurn, 1994). Taken together, the evidence indicates that PKC is involved in the slowly developed and maintained tension uniquely characteristic of contractile function in smooth muscle.

While it is clear that PKC may play an important role in the contractile response of vascular smooth muscle to various agonists, the exact mechanism by which this is accomplished remains uncertain. Rokolya et al, (1991) have shown that myosin light

chain phosphorylation levels increased upon the addition of phorbol ester to tissues precontracted with high potassium. During sustained potassium contractions, intracellular free calcium levels decline (Murphy et al, 1990) and myosin light chain kinase, which requires calcium for its activity, may be inactivated. Interestingly, even though PKC has been shown to phosphorylate MLC at sites distinct from those phosphorylated by MLCK, the phosphorylation of MLC observed with phorbol ester addition to potassium contracted tissues was consistent with MLCK activity. This suggests that under some conditions PKC may play a direct or indirect role in the activation of MLCK. There is also evidence that PKC phosphorylates a number of cytoskeletal intermediate filament and regulatory proteins (Rasmussen et al., 1987). It was proposed that these phosphorylations result in a stabilization of the cytoskeletal domain containing filamin, actin and desmin which could contribute to the maintenance of active force. Another possibility is that PKC exerts its influence on smooth muscle via its effects on mitogen activated protein kinase. It has been shown that MAP kinase undergoes a concurrent translocation with PKC in freshly isolated ferret aortic smooth muscle cells (Kahlil and Morgan, 1993). Following an initial membrane directed movement, MAP kinase is transported back into the cytoplasm. This re-distribution of the MAP kinase could explain how activated PKC, which remains associated with the membrane during contraction, can exert an influence on the contractile elements located throughout the cell and how the activation of MAP kinase in smooth muscle may be accomplished.

Summary

While, at first glance, smooth muscle appears to be an uncomplicated tissue, the mechanisms of smooth muscle contraction remain, for a very large part, a mystery. Unlike striated muscle, where form follows function, smooth muscle lacks any apparent contractile mechanism but it does appear that there may be some degree of applicability of a modified version of sliding filament mechanics to smooth muscle contraction. According to this hypothesis, smooth muscle contraction is initiated by a rise in intracellular calcium produced by agonist stimulation or to depolarization. This rise in intracellular calcium concentrations allows for the association of calmodulin with calcium, and this calcium:calmodulin complex then binds to myosin light chain kinase, activating the enzymatic properties of the protein. This is the first step in the mechanical generation of force. Myosin light chain kinase, when turned on, acts to phosphorylate a serine residue on the myosin regulatory light chain. When phosphorylated, the light chain permits the association of the myosin head with an actin filament, this induces subsequent ATPase activity and crossbridge cycling. There are many characteristics of the smooth muscle contraction that do not fit neatly within the constraints of this process, however, there are also many points for differential regulation within the system.

The inconsistencies between the experimentally obtained facts of smooth muscle contraction and the sliding filament-like theories proposed to explain the process which

are most often addressed include lack of calcium dependence, low energy consumption and high force generation with low cellular myosin content. The lack of calcium dependence is explained by the presence of calcium sensitizing agents within the cell that are activated in response to certain contractile stimuli but not to others. Low energy requirements by actively contracting smooth muscle are interpreted within the framework of a mathematical model. Latch theory predicts that unphosphorylated myosin can maintain its attachment to actin, thus lowering the energy consumption for slowly developing and maintained contraction. However, experimental evidence for actin binding by this unphosphorylated myosin *in vivo* is lacking. The cooperative theory of myosin binding could possibly explain the low myosin light chain phosphorylation levels found with contraction, but is hard to verify beyond conjecture and extrapolation. That smooth muscle can develop at least as much tension as striated muscle with fractional levels of cellular myosin content is examined as a function of the myosin molecule itself. The myosin of smooth muscle may have a longer duty cycle, enabling longer periods of attachment, or it may not. Low myosin contraction could also be due to smooth muscle myosin being arranged into different type filaments that striated muscle myosin, thus allowing for longer filaments and greater actin:myosin interaction. The problem with many of these theories is that they are narrow in scope. Each of the proposed explanations for smooth muscle contraction addresses only one particular contractile property and, in each case, proposed mechanisms for explaining smooth muscle contractile properties are couched in terms of the existing framework of sliding filament theory while ignoring other systems in the smooth muscle cell that may play a

considerable role in the generation and maintenance of smooth muscle contraction.

Recent work investigating the role of the smooth muscle cytoskeleton in contraction is beginning to suggest that the examination of aspects of this system beyond those commonly acknowledged to be a part of the contractile process may give increased insight into the exact mechanisms of smooth muscle contraction. With evidence for the involvement of many varied cytoskeletal systems, it seems unlikely that the non-actin:myosin cytoskeleton does not play an important role in the contraction. The dynamic actin cytoskeleton has been shown to play an integral role in slow phase contraction and to be the location of numerous accessory intermediate filament proteins such as filamin and desmin. In addition, the microtubular cytoskeleton is beginning to emerge as a potential component of the smooth muscle contractile machinery. Many elements of the cytoskeleton have also been identified as either targets of phosphorylation during the course of the contraction or as a transducing elements in the activation and action of kinases.

Protein kinases of smooth muscle, other than myosin light chain kinase, are also beginning to be recognized as important potential players in the smooth muscle contraction. For example, several kinases, including calcium dependent calmodulin kinase, which have direct effects on the regulation of the established elements of contraction, along with other kinases are now being recognized to have effects via other pathways. Mitogen activated protein kinase is established as playing a role in contraction

as its inhibition produces effects on the force developed by smooth muscle. However, the exact mechanism of its actions are not clear. In addition, protein kinase C is widely recognized to be directly involved in smooth muscle contraction. Inhibition of this enzyme has significant effects on contraction, but its exact mode of action is uncertain. PKC has been shown to be capable of phosphorylating myosin light chain and myosin light chain kinase and has also been shown to potentially play a role in the regulation of the MAP kinase cascade. It may be via one or all of these pathways that PKC exerts its effects, or it may be through another route entirely.

In order to fully understand the mechanisms of smooth muscle contraction, the importance of and the role for every system within the cell must be accounted for. Work is now progressing toward this end, and promises to bring an understanding to the mechanisms underlying smooth muscle contraction that extend beyond sliding filament theory.

II. Methods

Research Goals. The primary goal of this work was to better understand the mechanisms underlying vascular smooth muscle contraction and the regulation of these mechanisms. This was accomplished by: 1) examining the role of the non-actin:myosin cytoskeleton in vascular smooth muscle contraction; 2) assessing the importance of several kinases in the contractile process, and 3) integrating the knowledge gained concerning these two systems of the smooth muscle cell.

Examination of the non-actin:myosin cytoskeleton was accomplished in a series of experiments examining the effects of manipulation of the actin and microtubule components on smooth muscle contractile properties. Because it became apparent that the microtubules played an important role in slow force development by smooth muscle, further work was conducted to investigate the role of the microtubule associated motor protein kinesin in the contractile process. Because there are no pharmacological inhibitors available that specifically inhibited kinesin activity, antisense oligonucleotides were utilized to reduce the amount of kinesin protein produced in cultured tissues. Additional work included an *in vivo* analysis of the effects of antisense inhibition of kinesin synthesison the contractile properties of smooth muscle.

The roles of the various kinases of smooth muscle were examined in a series of contractility experiments utilizing pharmacological inhibitors specific to the targeted

kinases. These studies indicated PKC as being important to development of the slow component of contraction. Protein kinase C must be translocated from its resting location in the cytoplasm to cellular membranes for its full enzymatic activation. Because the microtubules are involved in the directed transport of many cellular components, and because the effects of PKC inhibition and microtubular disruption produced similar effects on smooth muscle contraction, it was reasoned that the microtubules may be acting as a transport system for the directed translocation of PKC following agonist stimulation. The viability of this hypothesis was examined by observing PKC translocation in response to phorbol ester and the changes in its translocation incurred by disruption of the cytoskeleton.

Animals. Twelve week-old male Sprague Dawley rats (Hilltop Laboratories, Scottsdale, PA) were used for all experiments. Animals were maintained at an ambient temperature of 23°C ±2°C and a 12:12hr light:dark cycle. Purina Rat Chow and fresh tap water were freely available.

The Effects of Cytoskeletal Disruption on Smooth Muscle Contractility

The initial goal for this series of experiments was to expand upon previous work which suggested a role for the dynamic actin cytoskeleton in smooth muscle contraction. The current work examined the functional and mechanical implications of cytoskeletal actin

disruption. Because this work confirmed a role for the non-actin:myosin cytoskeleton in smooth muscle contraction, a second part of the smooth muscle cytoskeleton, the microtubular portion, was examined in a similar fashion. Based on the results of these experiments, the role of the microtubule associated motor protein kinesin on contraction was assessed.

Tissue Preparation. The evaluation of *in vitro* contractile responses was performed as previously described (Huang et al, 1988). Rats were anesthetized with ketamine:xylazine (21:9 mg/kg), exsanguinated by cardiac puncture, and the thoracic aortae were surgically removed and transferred immediately to warmed, oxygenated buffer, cleaned of adherent tissue, and cut into rings ~ 0.3 cm in width. Rings were mounted at 1 g of passive tension in 25 ml glass organ baths containing Krebs buffer [(in mM) 118 NaCl, 4.7 KCl, 1.5 $CaCl_2$, 25 $NaHCO_3$, 1.1 $MgCl_2$, 1.2 KH_2PO_4, and 5.6 glucose; pH 7.4] maintained at 37°C and aerated with 5% CO_2 in O_2. Tissues were allowed to equilibrate a minimum of 1.5 hr before reference contraction. Contractions were recorded using a force transducer (Grass FT03) connected to a Grass polygraph (7D). The analysis of the contractile response was performed as previously described (Wright and Hurn, 1994). The separation of the development of force into fast and slow components was based on the rate of increase in tension (Figure 2). The portion of the contractile response in which the rate of increase in tension exceeded 1.0 g/min. was designated the fast response.

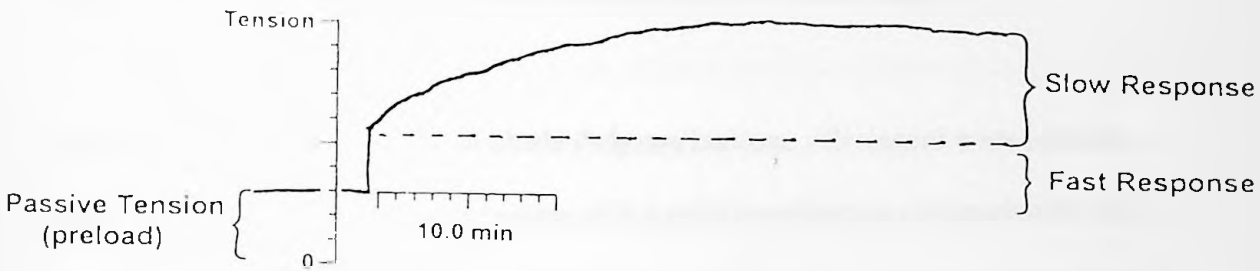

Figure 2 Representative tracing of K^+ induced smooth muscle contraction. Tissues were equilibrated at a passive tension of 1g prior to induction of contraction by the hypertonic addition of 80mM K^+. The contraction can be divided into two phases, an initial fast phase characterized by the rapid development of active tension, and a secondary slow phase in which active tension is developed at a slow rate and then maintained.

Passive Tension-Length Determinations. Tissues were excised and mounted as for contractile measurements. Following equilibration at 1 g of passive tension, the tissue lengths were measured using a micrometer caliper (Walter Stern, Inc., NY). The preload of each tissue was then raised from 1 g to 20 g in increments of 5 g. At each preload, the tissues were brought to a stable baseline and monitored for an additional 15 minutes to ensure maintenance of baseline prior to measurement of tissue length.

Inhibition of Actin and Microtubule Polymerization. All tissues were initially equilibrated for 2 h at a passive tension of 1 g prior to reference contraction by the addition of a hypertonic K^+ solution (80 mM) to the baths. Tissues were returned to baseline by washing with fresh buffer, and the passive tension was adjusted to preloads of 1, 5, 10, 15, or 20 g. Once the tissues maintained a stable baseline at the set preload, all tissues were again contracted by the addition of 80 mM K^+. To determine the effects of selective disruption of the microtubules or inhibition of actin cytoskeletal remodeling on active tension development at increasing passive tension preloads, colchicine (25 μg/ml) was added 20 minutes before contraction and cytochalasin B (2 μg/ml) was added 5 minutes prior to contraction. Because a large population of the microtubules are resistant to colchicine but are disrupted by cooling (Tsutsui et al., 1994), an additional experiment was conducted to examine the effect of this portion of the microtubular system on active tension. Tissues were excised and hung at 10 g passive tension for contractility measurements as described above. After a reference contraction to 80 mM K^+, tissues were rapidly (~ 1 minute) cooled to 0° C. Tissues remained at this temperature for 60

minutes, and then were quickly (~ 2 minutes) rewarmed to 37° C in the presence or absence of colchicine (25 μg/ml) with the drug added 20 minutes before rewarming began. Following return to 37° C, the tissues were reequilibrated for one hour and were then contracted with 80 mM K^+. Effectiveness of each treatment on disruption of the cytoskeleton was verified by microscopic analysis.

Microscopy. In order to verify the effects of actin and microtubule disrupting drugs, rat aortic smooth muscle cells in fifth through eighth passage were grown on glass coverslips until the cells had attached and begun to spread. The cells were untreated (vehicle) or were exposed to cytochalasin B or colchicine. To prepare for staining, the cells were fixed and permeabilized in ice cold acetone for one minute. For visualization of cytoskeletal components, fluorophore labeled primary antibodies (Sigma Immunochemicals) to smooth muscle actin and tubulin or TRITC labeled phalloidin were used. The preparations were mounted on a Nikon Diaphot microscope, and confocal microscopy was performed with a BioRad Model 1024 scanning system using a krypton/argon laser. Final micrographic images were built by projecting serial Z-plane image acquisitions and were analyzed using Lasersharp and Confocal Assistant software (BioRad, CA).

Oligonucleotide Synthesis and Preparation. Antisense and sense oligonucleotides directed toward the kinesin heavy chain were synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer. Each oligonucleotide incorporated phosphorothioate

modifications to aid in stabilizing the oligonucleotide by making it more resistant to nuclease degradation (Wagner et al., 1993). The antisense sequence used was GCCGGGTCCGCCATCTTTCTGGCAG (Amartunga et al., 1993). Oligonucleotides were purified by ethanol precipitation, resuspended in TE buffer quantified by A_{260} (pH 8.0), and stored at 4°C.

Blockade of Kinesin Protein Synthesis. Vessels were treated *in vitro* with the oligonucleotides to block kinesin protein synthesis. Tissues were cultured as previously described (Wright et al., 1996). Briefly, an aorta was excised, cut into rings as above, and the luminal surfaces of the rings were gently rubbed to remove the endothelial cell layer. The aortic rings were then transferred into Leibovitz's L-15 media (5 ml) supplemented with rat plasma (5 ml) and penicillin/streptomycin (1050 U/1050 µg) and incubated at 37°C in an atmosphere of 5% CO_2 in air for 24 hours. The oligonucleotides (1-2 umol in TE pH 8.0) were mixed with Lipofectin® (100 µl) prior to addition to the culture media. Lipofectin® acts to aid the passage of the oligonucleotide into the vessel wall by incorporating the oligonucleotide into liposomes. Plasma was added last to limit the incorporation of plasma components into the liposomes. Following a 24 hour incubation, tissues were mounted for contractility determinations and contracted at 1 g of passive tension with 80 mM K^+.

Immunoblot Analysis. In order to determine if the kinesin protein levels were reduced in response to the antisense oligonucleotide treatment, aortae were homogenized in

buffer (pH 7.5, 20 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, 2 mM PMSF) at 4°C. Protein concentrations were determined by the BCA®(Pierce) protein assay. Samples of equal protein concentrations were denatured in sample buffer (50 mM Tris, 10% glycerol, 20 mM DTT) before being subjected to 10% SDS-PAGE. Proteins were electrically transferred to Hybond® nitrocellulose membrane (Amersham). The membrane was incubated for an hour at room temperature in blocking solution (5% nonfat dry milk in phosphate buffered saline, pH 7.5, containing 0.5% Tween-20). A 1:2000 dilution of monoclonal anti-kinesin heavy chain antibody (Sigma) was added for 1 hour at room temperature. The blot was washed several times in PBS-0.5% Tween followed by a 1 hour incubation with a 1:1000 dilution of rabbit anti-mouse horseradish peroxidase conjugated secondary antibody. Following incubation, the blot was again washed with PBS-Tween. Immunoblots were visualized using the ECL Western blotting detection system (Amersham) and quantified by 2D-densitometric scanning (ImageQuant, Molecular Dynamics, Inc.). Protein levels were expressed in arbitrary units.

Statistics. Data are presented as mean ± SEM. All data comparisons for length-passive tension determinations were done by a one way analysis of variance followed by a Student Newman-Keuls test. All other comparisons were done by unpaired student's t-test. $P < 0.05$ was considered as statistically significant in all analyses.

Modification of previous methods for *In Vivo* Application of Antisense Oligonucleotides

In the *in vitro* preparations utilized for examination of the effects of antisense inhibition of kinesin protein synthesis, untreated control tissues were found to contain a significantly lowered kinesin concentration as compared to fresh tissues. In order to study the effects of inhibition of kinesin synthesis on tissues which contained a full complement of kinesin protein levels, it became important to utilize an *in vivo* method. The method most commonly used for *in vivo* transfer of oligonucleotides called for the incorporation of the oligonucleotides into a detergent-like pluronic gel. The characteristics of pluronic gels make them unsuited for examination of smooth muscle functions because even short-term application of these compound causes massive increases in plasma cholesterol levels. This is significant to the study of smooth muscle contraction because elevated plasma cholesterol levels cause potentiation of the contraction and consequently; an inert delivery media was needed. Knox gelatin was used for application of antisense oligonucleotides since it did not raise plasma cholesterol levels or have significant effects on the contraction.

Preparation of Packing Vehicle. For studies using pluronic gel, the pluronic gel F127 powder (BASF) was mixed with cold water at a concentration of 25%. Preparation of the gelatin vehicle was accomplished by first adding a small amount of room temperature distilled deionized water (ddH_2O) (100 ul) to 0.04 g of gelatin, since this swells the

gelatin and improves its solubility. After several minutes, 300 ul boiling ddH_2O were added and the mixture gently agitated until the gelatin was completely dissolved. When the gelatin was completely in solution, 250 ul room temperature ddH_2O was added and the mixture was put on ice briefly to cool.

Incorporation of Compounds into Packing Vehicle. For oligonucleotide incorporation into pluronic gel, 8 umoles sense or antisense oligonucleotide was added to the mixture before it began to gel. For addition of oligonucleotides to the gelatin vehicle, 8 uMol sense or antisense oligonucleotide was first mixed with 50 uL Lipofectin® and incubated for several minutes to allow liposomes to form. This mixture was then added to the gelatin after it had reached room temperature, but before it had begun to solidify. Phorbol 12,13 dibutyrate (10^{-6} M) and β-galactosidase containing plasmid (100 ug) were incorporated in the same manner.

Application of Pluronic Gel or Gelatin Vehicle. The vehicle containing kinesin sense or antisense oligonucleotides, PDB or plasmid encoding *β*-galactosidase was applied to aortae *in situ* by a modification of the method of Bennett et al. (1994). Rats were anesthetized with ketamine:xylazine (21:9 mg/kg). The abdomen was opened, the vehicle containing the compound to be studied or vehicle alone was packed around the abdominal aorta, and the preparation was covered with a square of polyvinyl-chloride film prior to replacement of the intestines. Following this procedure, the rats were allowed free access to Purina Rat Chow and tap water. After 24 hours, the abdominal aortae were removed

and prepared for contractility measurements.

Tissue preparation. The evaluation of *in vitro* contractile response was performed as described above (Pg. 40) except for that the abdominal aortae were utilized in these experiments.

Immunoblot Analysis. Determination of the effectiveness of the *in vivo* application of kinesin antisense oligonucleotides was performed the same as for the determination of kinesin levels in *in vitro* studies as described above (Pgs. 44-45)

Statistics. Data were presented as mean ± SEM. Comparisons were done by unpaired Student's t-test. $P < 0.05$ was considered as statistically significant in all analyses.

The Effects of Kinase Inhibitors on Smooth Muscle Contraction

Because there is an established role for many kinases in the generation and maintenance of active force in smooth muscle, and because many of these kinases exert their effects either directly or indirectly on the components of the cytoskeleton, a panel of specific kinase inhibitors were examined to assess their effects on the fast and slow components of contraction.

Tissue preparation. The evaluation of *in vitro* contractile responses was performed as described above (Pg. 40).

Inhibition of Kinase Activity. The effect of inhibition of enzyme activity of several kinases reported to be present in smooth muscle was examined by the application of specific inhibitors to the contractile baths. For most inhibitors, concentrations of 10 percent of IC_{50} (as determined by the manufacturer), 100 percent of IC_{50} and 1000 percent IC_{50} were applied and allowed to remain in the baths 30 minutes prior to contraction with 80 mM potassium. For staurosporine inhibition of PKC, concentrations of 3, 6, and 30 nM were used in the same manner.

Statistics. Data were presented as mean ± SEM. Comparisons were done by unpaired Student's t-test. $P < 0.05$ was considered as statistically significant in all analyses.

Evaluation of the Interactions Between the Cytoskeleton and Protein Kinase C

Based on the results from previous experiments, PKC was selected for further study. It is well established that PKC must be translocated from its resting position in the cytosol to cellular membranes to be fully enzymatically activated. Because the microtubules are responsible for the transport of many cellular components, and because the inhibition of

slow phase contraction produced by microtubular disruption is similar to that caused by inhibition of PKC activity, a possible role for the microtubules in the translocation of PKC was examined. Initially, cell fractionation and Western blot analysis were utilized for this purpose. However, because the resolving power of this type of analysis is limited to discerning between only a particulate and a cytosolic fraction, a more powerful technique was essential. The use of confocal microscopic techniques allowed for visualization of PKC movements in response to phorbol ester stimulation, and the effects of cytoskeletal disruption on this process were examined.

Cell Culture. Aortae were aseptically removed from 100 g male rats and the cells were enzymatically dispersed in a cocktail of collagenase, elastase, DNAse, soybean trypsin inhibitor and bovine serum albumin (BSA) in Hanks Buffered Saline Solution (HBSS) containing calcium. Dispersion was carried out in a 37° C shaker bath for 60 minutes. Following dispersion, cells were strained through a fine mesh to filter out residual connective tissue, and the cells were centrifuged to remove the digesion media. The cell pellet was then resuspended in serum-free Dulbecco's Modified Eagle Media (DMEM). This cell suspension was plated into a 25 mm^2 tissue culture flask and allowed to incubate for 2 hours to allow cell attachment. After this initial period of attachment, DMEM containing 10% fetal calf serum was added. Cells were maintained at 37° C in an atmosphere of 5% CO_2 in air. Upon reaching confluence, cells were detached from the plate with a trypsin/EDTA solution in HBSS and were split into 2 or more flasks depending upon the number of cells. This procedure was repeated at regular intervals as

cells reached confluence. Only cells in passage two through six were utilized for experiments.

PKC Translocation. Translocation of protein kinase C was initiated by the addition of 10^{-6} M phorbol 12,13 dibutyrate (PDB) for 10 minutes to tissues or to cells in culture. Cultured smooth muscle cells were isolated from rat aorta and utilized in passages 2-6. To assess their effects on translocation, colchicine (40 ug/ml, 20 minutes) or cytochalasin B (1 ug/ml, 5 minutes) was added prior to phorbol stimulation. For the combined cold plus colchicine treatment, tissues or cells were maintained on ice for 60 minutes with colchicine (40 ug/ml) added at 40 minutes. At the end of the cooling interval, the samples were rewarmed to 37^0C and equilibrated for an additional 60 minutes before the addition of PDB.

Immunoblot Analysis. In order to determine PKC levels in the cell soluble and particulate fractions, fresh aorta or cultured cells were homogenized in lysis buffer (20 mM Tris, pH 7.5; 0.5 mM EDTA; 0.5 mM EGTA; 25 ug/mL aprotinin; 25 ug/mL leupeptin) at 4°C. Cells were washed once with ice-cold lysis buffer and were then homogenized by several passages through a 21-gauge needle. These homogenates were centrifuged at 100,000g for 60 min at 4°C and the supernatant was reserved as the cytosolic fraction. The pellet was re-solubilized in lysis buffer containing 0.5% Triton X-100, and the resulting suspension was maintained on ice for 40 minutes. The suspension was then re-centrifuged at 100,000g for 60 min at 4°C, and the supernatant analyzed as

the particulate or membrane fraction. Protein concentrations were determined by the BCA®(Pierce) protein assay. Samples of equal protein concentrations were denatured in sample buffer (50 mM Tris, 10% glycerol, 20 mM DTT) before being subjected to 10% SDS-PAGE. Proteins were electrically transferred to Hybond® nitrocellulose membrane (Amersham) and the membrane incubated for 1 hour at room temperature in blocking solution (5% nonfat dry milk in phosphate buffered saline, pH7.5, containing 0.5% Tween-20). A 1:2000 dilution of monoclonal anti-PKC α antibody (UBI) was then added for 1 hour at room temperature. Detection of protein was performed as described above (Pg. 45).

Microscopy. Rat aortic smooth muscle cells in the second through sixth passage were grown on glass coverslips until the cells had attached and begun to spread. The cells were untreated (vehicle) or were exposed to cytochalasin B or colchicine and were then left in the presence or absence of PDB for 30 seconds to 10 minutes. To prepare for staining, the cells were fixed and permeabilized in ice cold acetone for one minute. For immunolocalization of PKC, this was followed by incubation with antibody specific to PKC-α (UBI) for 30 minutes at room temperature followed by washing in PBS, and the addition of an FITC labeled secondary antibody for 30 minutes at room temperature. For co-localization studies, PKC-α was visualized with a Texas Red labeled secondary antibody (Molecular Probes) and tubulin by a FITC labeled primary antibody. The preparations were mounted on a Nikon Diaphot microscope, and confocal microscopy was performed with a BioRad Model 1024 scanning system using a krypton/argon laser.

Final micrographic images were built by projecting serial Z-plane image acquisitions and were analyzed using Lasersharp and Confocal Assistant software (BioRad, CA).

Statistics. Data were presented as mean ± SEM. Comparisons were done by unpaired Student's t-test. $P< 0.05$ was considered as statistically significant in all analyses.

III Results

The Effects of Cytoskeletal Disruption on Smooth Muscle Contractility

With the tissues held at passive tensions of 1 g to 20 g, the relationship between the preload and the tissue length was nearly linear (Figure 3). Within this range of passive tension preload, there were marked differences in the fast and slow components of the active tension response to potassium depolarization of the tissue (Figure 4). The fast component of active tension development was significantly increased by increasing the preload from 1 g to 5 g and subsequent increases in the preload from 5 g to 15 g had no further effect on the magnitude of the fast component. However, increasing the preload from 15 g to 20 g resulted in an approximate 50% reduction in the fast response as compared to the peak value obtained at 10 g of passive tension. By comparison, the slow component of the active tension response showed no effect of increasing the preload up to 10 g. At higher preloads, the slow response fell sharply and was approximately 30% of the peak value when stretched to 20 g of passive tension.

In order to assess the role of the cytoskeleton on the relationship between passive tension preload, tissue length, and active tension development, the drugs colchicine and cytochalasin B were used to selectively inhibit the microtubule and actin-containing cytoskeletal components, respectively. Colchicine has been demonstrated to cause disassembly of actively polymerizing microtubules, whereas the cytochalasins bind the

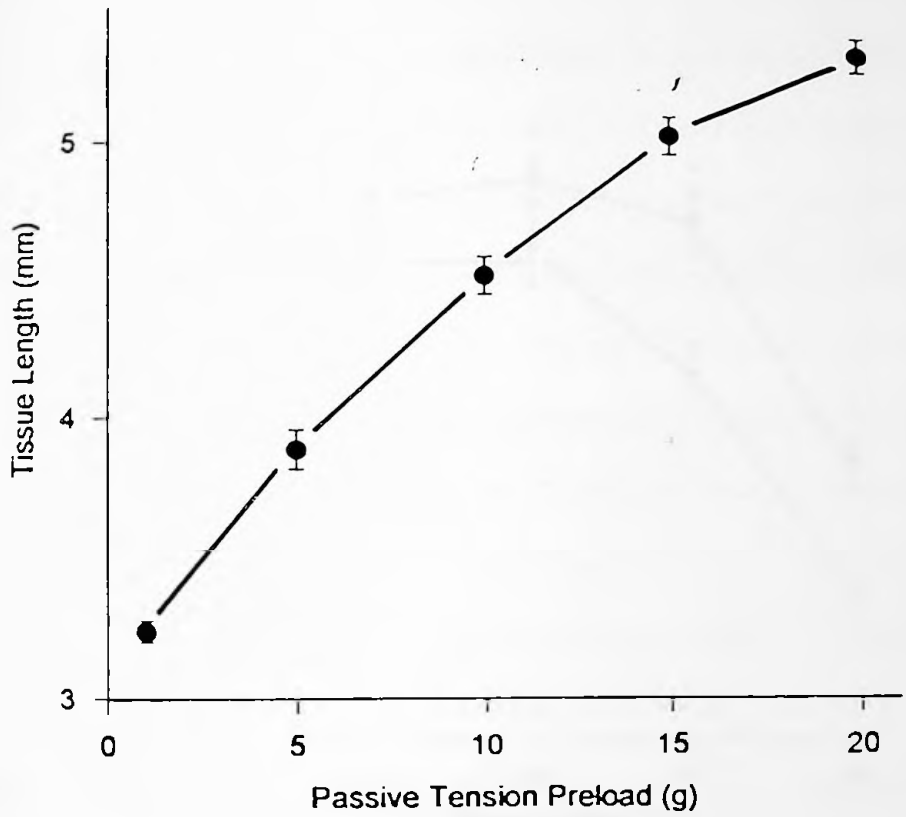

Figure 3 Length - passive tension relationship of untreated control tissues. Each point is an average of 32 tissues.

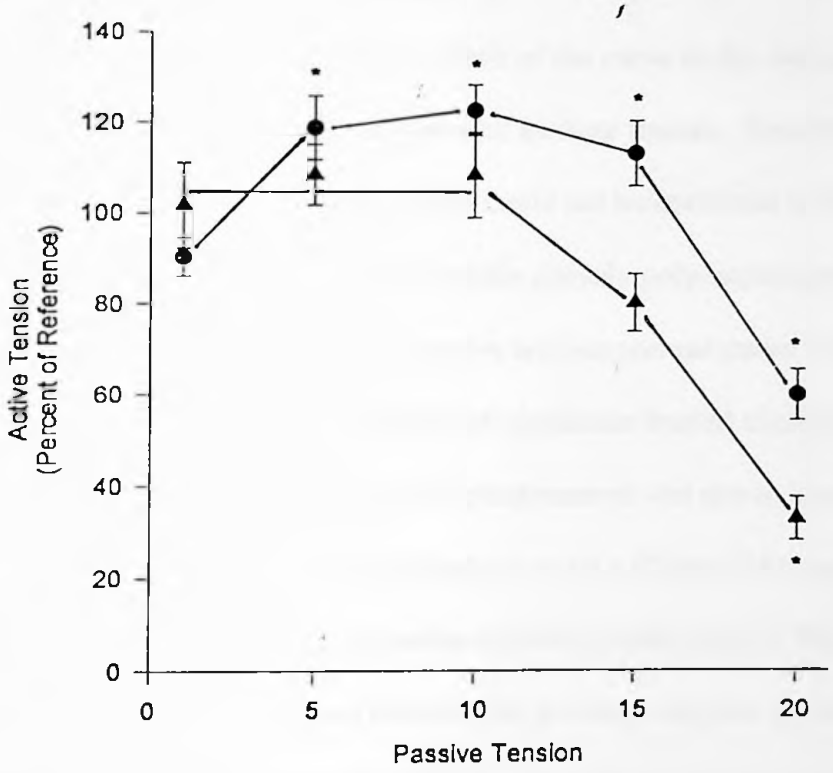

Figure 4 Effect of increasing passive tension on active tension development in freshly excised aortic rings. Contraction is divided into fast (●) and slow (▲) components. Active tension is presented as percent of 1g reference contraction. Pooled reference values are fast :1.08 ± 0.03g, slow:1.77 ± 0.05g. Each point represents average of values from 12 tissues. * indicates a significant difference ($p < 0.05$) from 1 g values of same component.

barbed end of actin and prevent dynamic remodeling of the actin cytoskeleton. The treatment of tissues with cytochalasin B had no observable effect on the relationship between passive tension and tissue length (Figure 5). By comparison, the exposure of the tissues to colchicine at 37°C resulted in a shift of the curve to the right, indicating a slight increase in the mechanical resistance to stretch in these tissues. Because tissues weights were identical between groups, these results could not be attributed to differences in the size of the tissues. Colchicine disruption of the actively polymerizing microtubules resulted in a shift of the active tension-passive tension preload curve to the left (Figure 6A). The resolution of the active response of colchicine-treated tissues into their fast and slow components further revealed that this phenomenon was due to a marked enhancement of the fast response at preloads up to 10 g (Figure 7A), and a reduction in the slow response at 10 g to 15 g of passive tension preload (Figure 7B). By comparison, cytochalasin B caused a significant reduction in the active tension response at each tissue preload studied (Figure 6B), and this effect was due almost exclusively to a reduction of the slow response (Figure 8).

There appears to be a large population of microtubules that are insensitive to colchicine but that may be depolymerized by cooling to 0°C for 60 minutes (Tsutsui et al., 1994). Because colchicine exerts its effects on actively polymerizing microtubules, it was reasoned that by adding colchicine prior to the rewarming of the tissues and the subsequent repolymerization of the microtubules, the effect of the dissolution of this more stable microtubule population on contraction could be observed. The fast

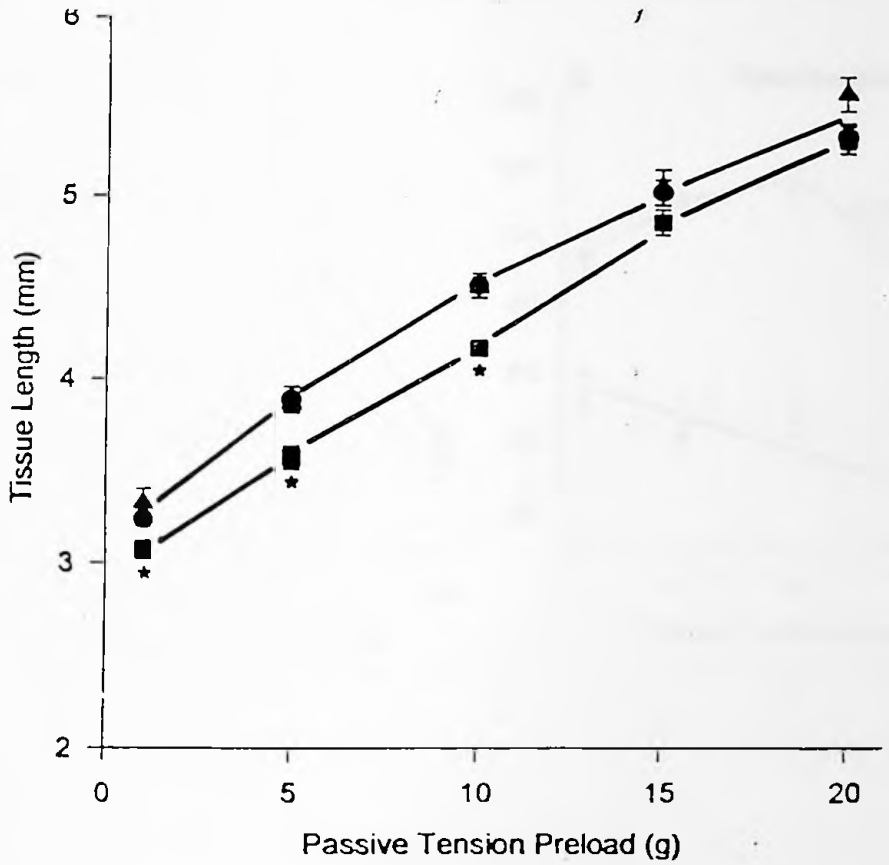

Figure 5 Length - passive tension relationship of tissues treated with colchicine (■) or cytochalasin B (▲) compared to untreated controls (●). Each point is an average of 16 tissues. * indicates a significant difference ($p < 0.05$) from control at given preload.

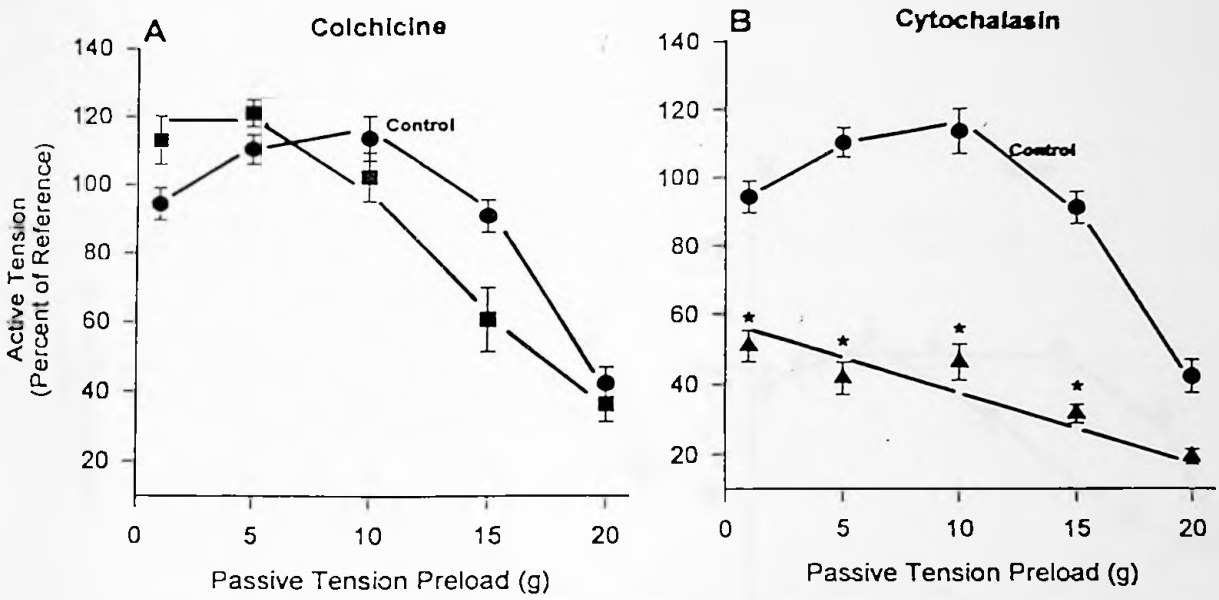

Figure 6 Total tension developed in response to high potassium (80 mM) at increasing preload. (A) Disruption of microtubules by colchicine (■) as compared to control (●). (B) Inhibition of actin polymerization by cytochalasin B (▲) as compared to control (●). All data are presented as percent of reference contraction at 1 g. Pooled reference values for colchicine; 2.47 ± 0.12 g, and cytochalasin B: 2.43 ± 0.09 g. Each point represents the average of 8 to 13 tissues. * indicates a significant difference ($p < 0.05$) from control values at given preload.

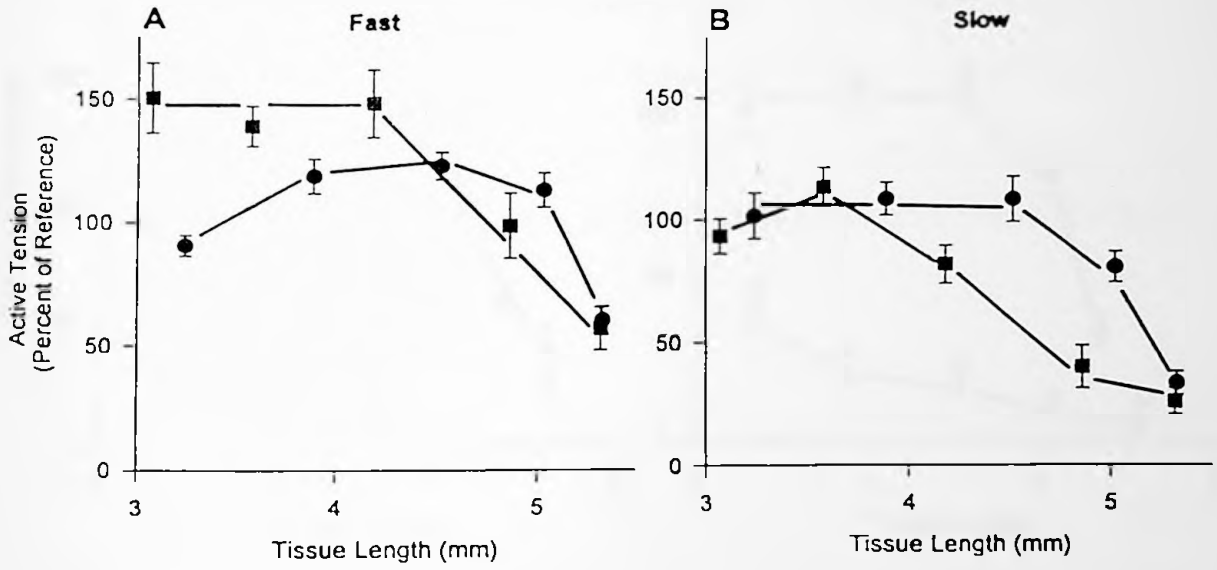

Figure 7 The (A) fast and (B) slow components of contraction with increasingtissue length in control (●) and colchicine-treated (■) aortic rings. Data are calculated as percent of reference contraction at 1g. Pooled reference values: fast, 0.88 ± 0.05g; slow, 1.59 ± 0.08g. Each point represents the average of 10 to 12 tissues.

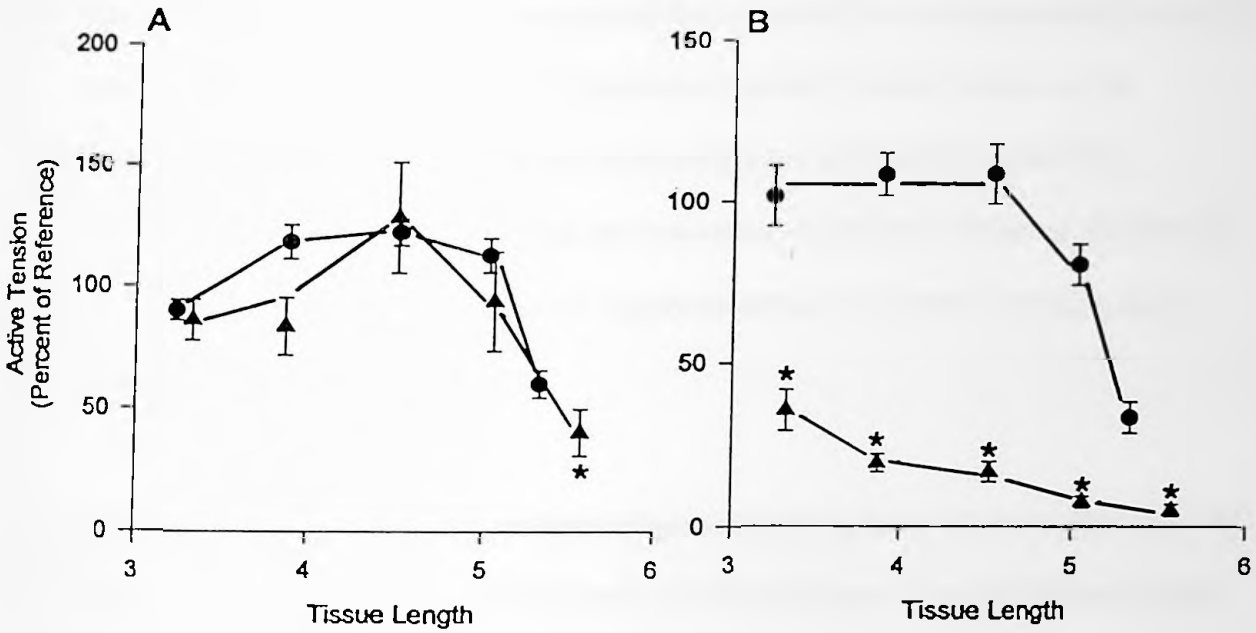

Figure 8 The (A) fast and (B) slow components of contraction in control (●) and cytochalasin B-treated (▲) aortic rings. Data are calculated as a percent of reference contraction at 1 g. Pooled reference values: fast 0.80 ± 0.03 g, slow 1.63 ± 0.07 g. Each point represents the average of 8 to 13 tissues. * indicates a significant difference ($p < 0.05$) from control tissue.

component of the contractile response of tissues that were cooled and rewarmed was significantly increased; whereas, the slow component of the response was identical to control tissues which were maintained at 37° C throughout the experiment (Figure 9). The colchicine-treated tissues that were cooled and rewarmed, however, showed a significant reduction in the slow component of contraction but no significant effect on the fast component of active tension development compared to control indicating the effective reduction of both the fast and slow component compared to cooled and rewarmed tissues (Figure 9). Cooling and rewarming of the tissues in either the presence or absence of colchicine did not have a significant effect on the passive tension-tissue length relationship (Figure 10).

Incubation of aortic rings with antisense oligonucleotides to kinesin heavy chain reduced the amount of kinesin protein by an average of 35% compared to sense oligonucleotide-treated tissues, as determined by SDS-PAGE analysis (Figure 11). The reduction of kinesin heavy chain protein synthesis by the incubation of aortic rings with antisense oligonucleotide *in vitro* resulted in the reduction of the contractile response to potassium by about 35% as compared to control tissues. The inhibition of response was evenly distributed between the fast and slow components (Figure 12). The results obtained from tissues exposed to kinesin heavy chain sense and antisense oligonucleotides *in situ* were effectively identical to those incubated with the oligonucleotides in organ culture (data not shown). These results could not be attributed to an effect of the antisense oligonucleotide on the mechanical properties of the tissue (Figure 13). The tissue length-

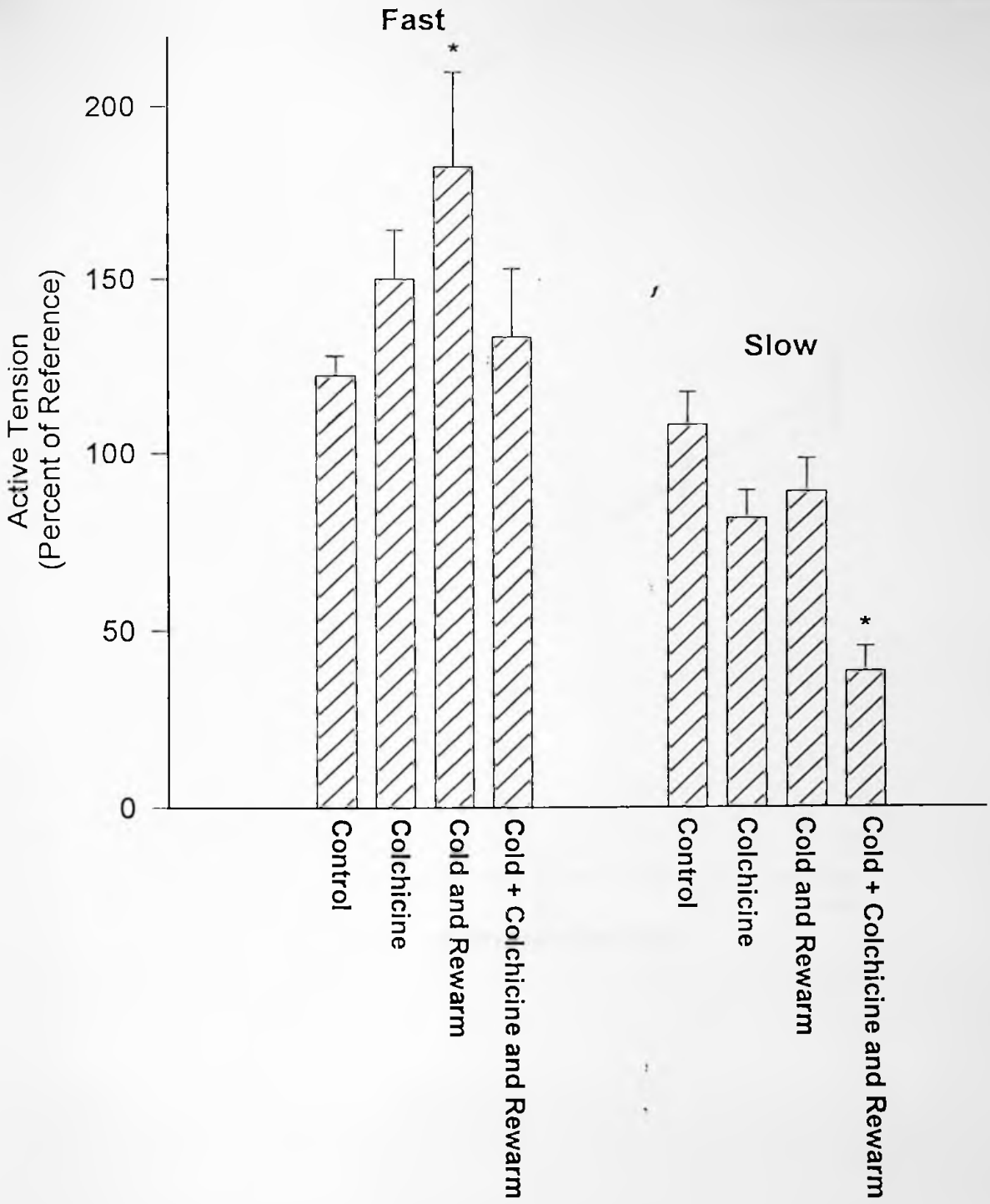

Figure 9 Effects of cold temperature and rewarming in the presence or absence of colchicine on the fast and slow components of the contractile response to 80mM K^+. Values represent the average of 5 to 9 tissues. Data are presented as percent of reference (Fast = 0.90 ± 0.05g, Slow = 2.29 ± 1.23g. An asterisk indicates a significant difference from control tissues that were not cooled but were maintained at 37°C throughout the experiment. * indicates a significant difference ($p < 0.05$) from control tissue.

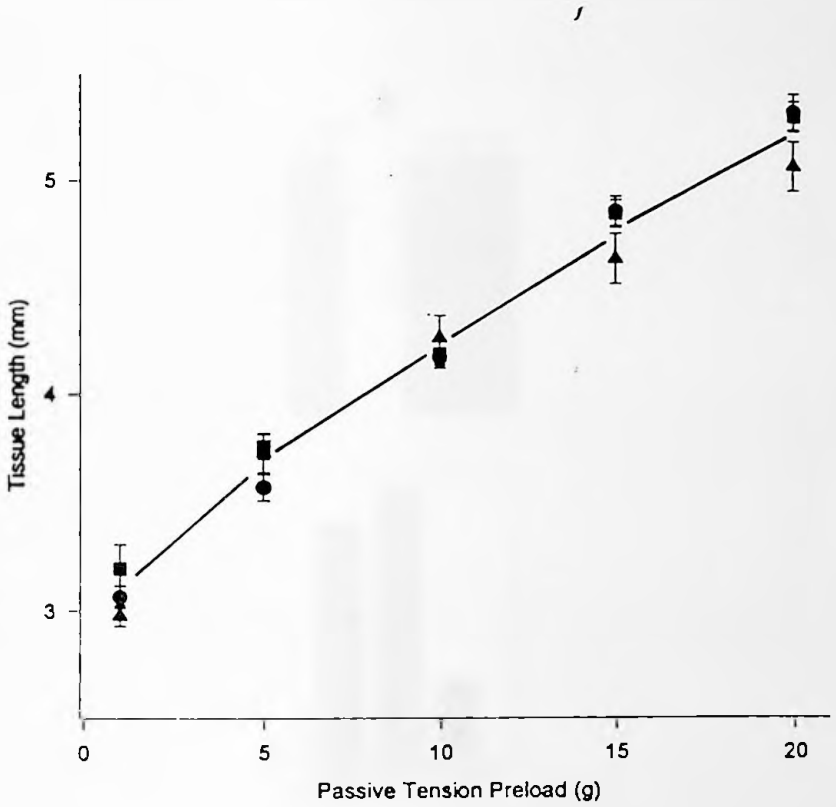

Figure 10 Length - passive tension relationship of cooled and rewarmed tissues treated with colchicine. Colchicine (●), cold-treated control (■), cold-treated+colchicine (▲). Each point is an average of 7 to 9 tissues.

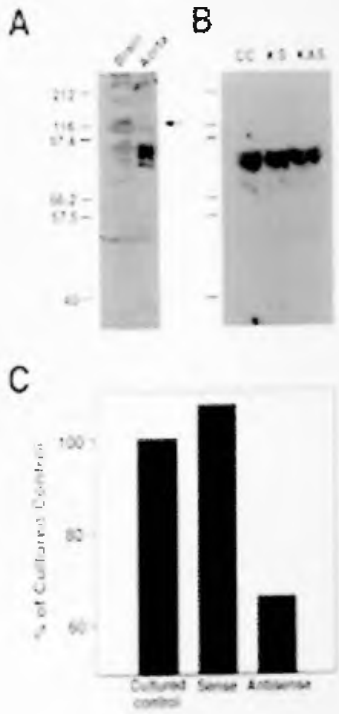

Figure 11 SDS-PAGE analysis by Western blot. (a) Freshly excised aortic and brain preparations (b) cultured aortic tissues; 24 hour cultured control (CC); 24 hour cultured in the presence of kinesin heavy chain sense oligonucleotides (KS); 24 hour cultured in the presence of kinesin heavy chain antisense oligonucleotide (KAS). (c) Bar graph showing the densitometric analysis of protein bands. Molecular weight markers are indicated. Arrow indicates kinesin heavy chain.

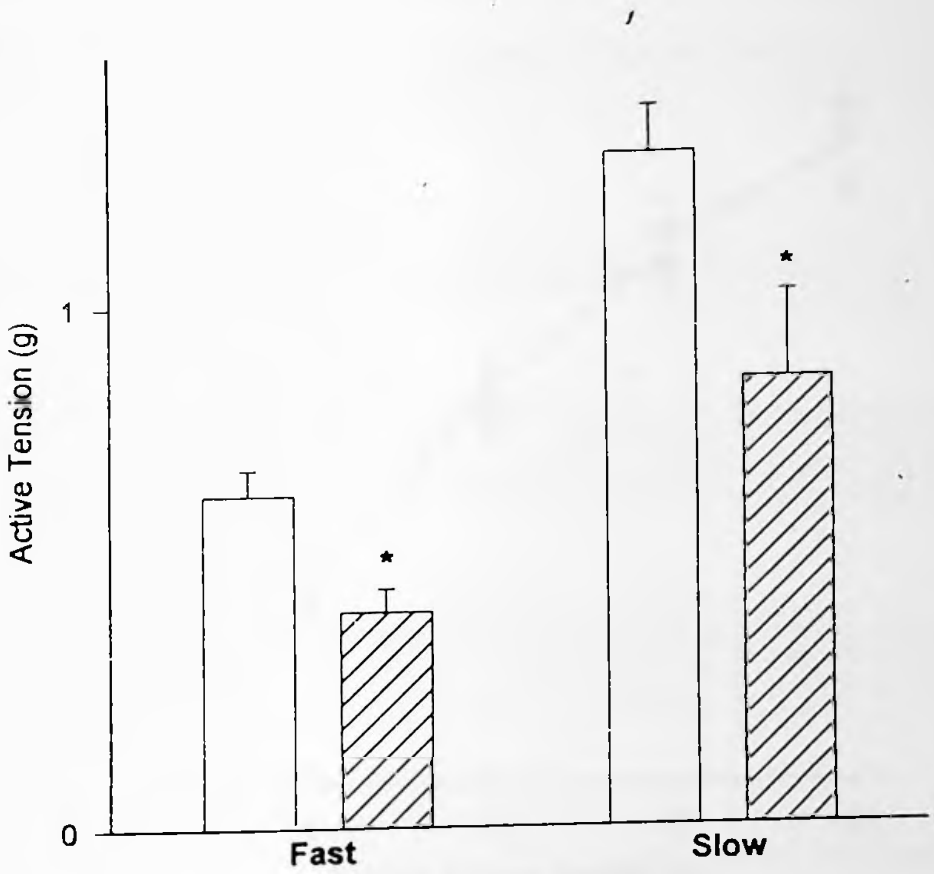

Figure 12 Effects of the kinesin antisense (hatched bar) and corresponding sense control (open bar) oligonucleotides on the fast and slow components of contraction. Aortic rings were incubated in organ culture with oligonucleotides for 24h prior to contractions at 1 g of passive tension. Each bar represents an average of 14 to 19 tissues. An asterisk indicates a significant difference ($p < 0.05$) between sense and antisense groups.

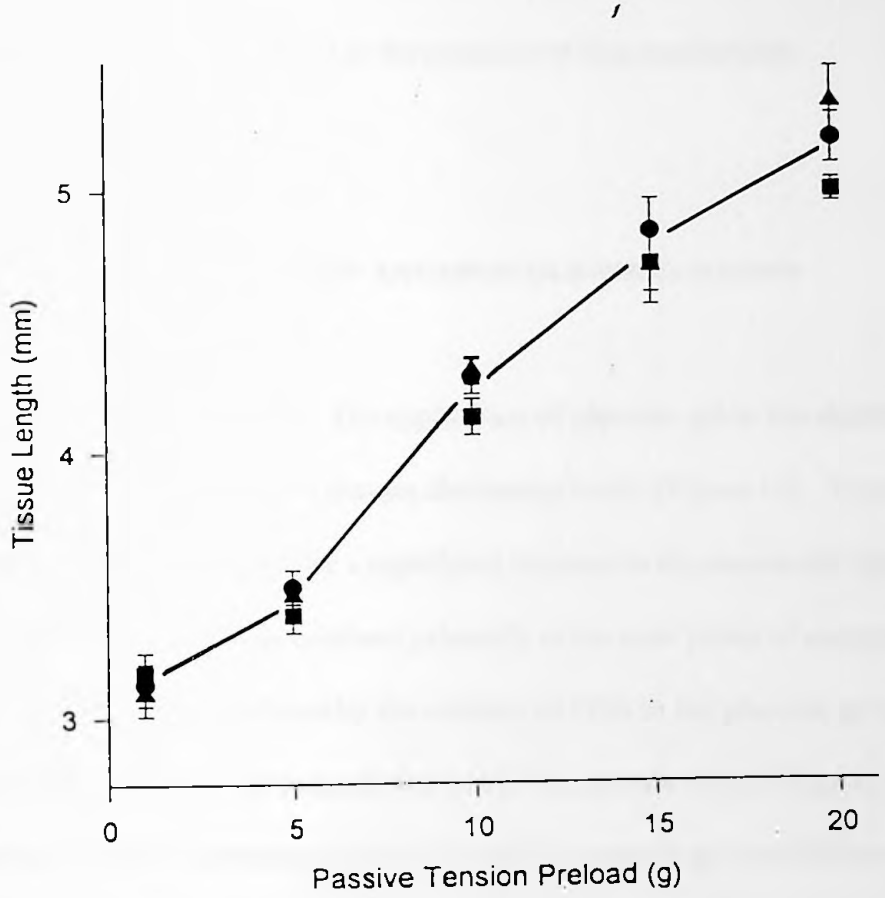

Figure 13 Length - passive tension relationship of tissues incubated with kinesin antisense oligonucleotide (■) as compared to untreated control tissues (●) and those incubated with sense oligonucleotide (▲). Each point represents the average of 4 tissues.

passive tension preload curve of aortic rings maintained in organ culture for 24 hours was shifted to the right as compared to that of freshly excised rings. However, there were no significant differences in this length-passive tension relationship of control aortic rings in culture and those tissues cultured in the presence of oligonucleotides.

In Vivo Application of Kinesin Antisense Oligonucleotides

Effects of Pluronic Gel F127 The application of pluronic gel to the abdominal aorta produced a dramatic increase in plasma cholesterol levels (Figure 14). These increases in cholesterol were accompanied by a significant increase in the contractile response of the tissues, and this increase was confined primarily to the slow phase of contraction (Figure 15). This rise could be inhibited by the addition of PDB to the pluronic gel prior to application (Figure 15); however, cholesterol levels remained high (Figure 14). The application of kinesin antisense oligonucleotides in pluronic gel had little to no effect on the contraction (Figure 15).

Effects of Gelatin Because of the unphysiological plasma cholesterol levels induced by the pluronic gel, and because many cellular functions, including smooth muscle contraction, are affected by high cholesterol, an alternative vehicle for oligonucleotide delivery was used. A biologically inert gelatin compound was employed as a vehicle for the same compounds previously used with the pluronic gel. To test whether this method

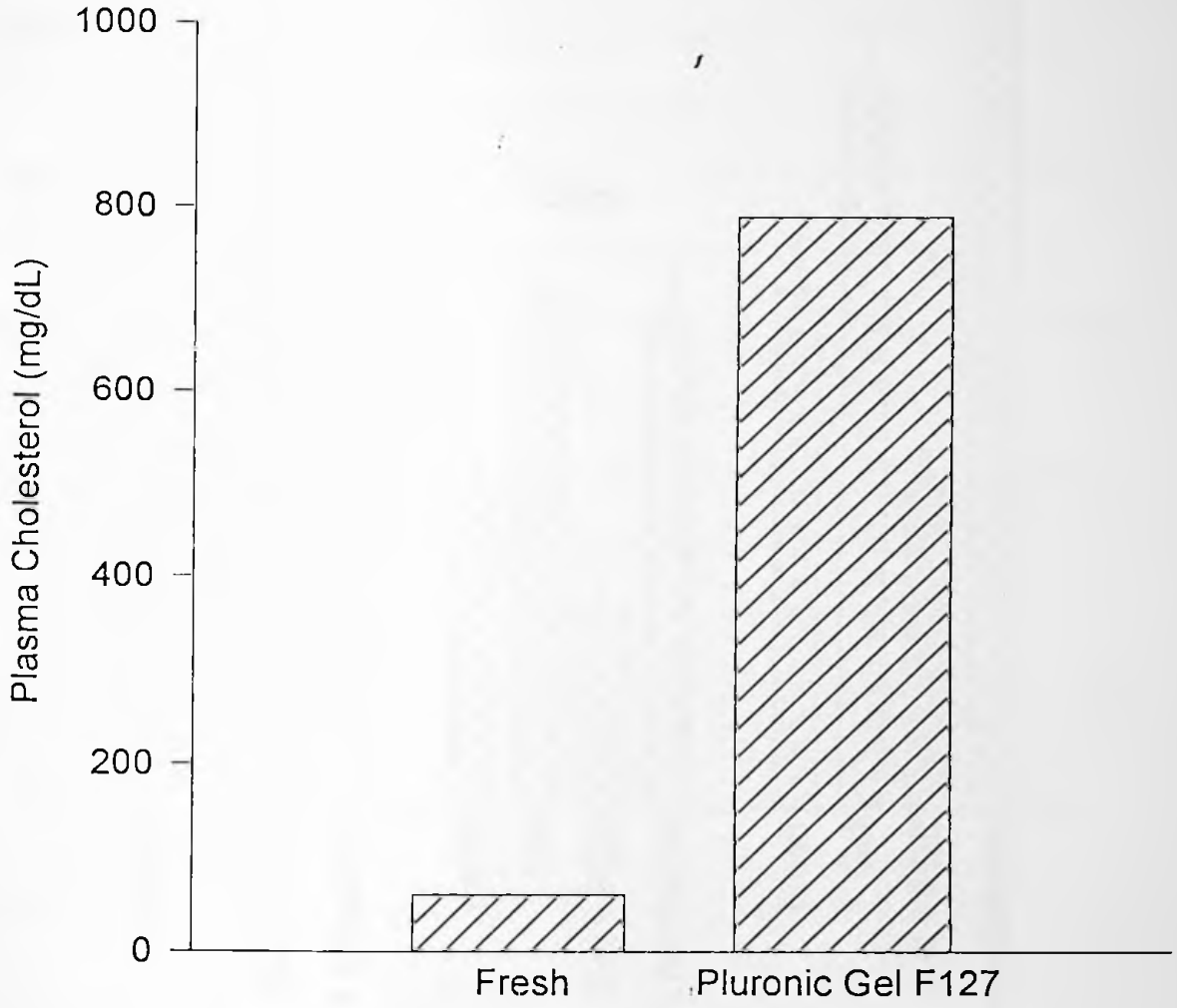

Figure 14 Effect of *in vivo* application of pluronic gel F127 on plasma cholesterol levels. Twenty four hour exposure produced approximately a ten-fold increase over control levels. Values represent pooled plasma from 3-5 rats.

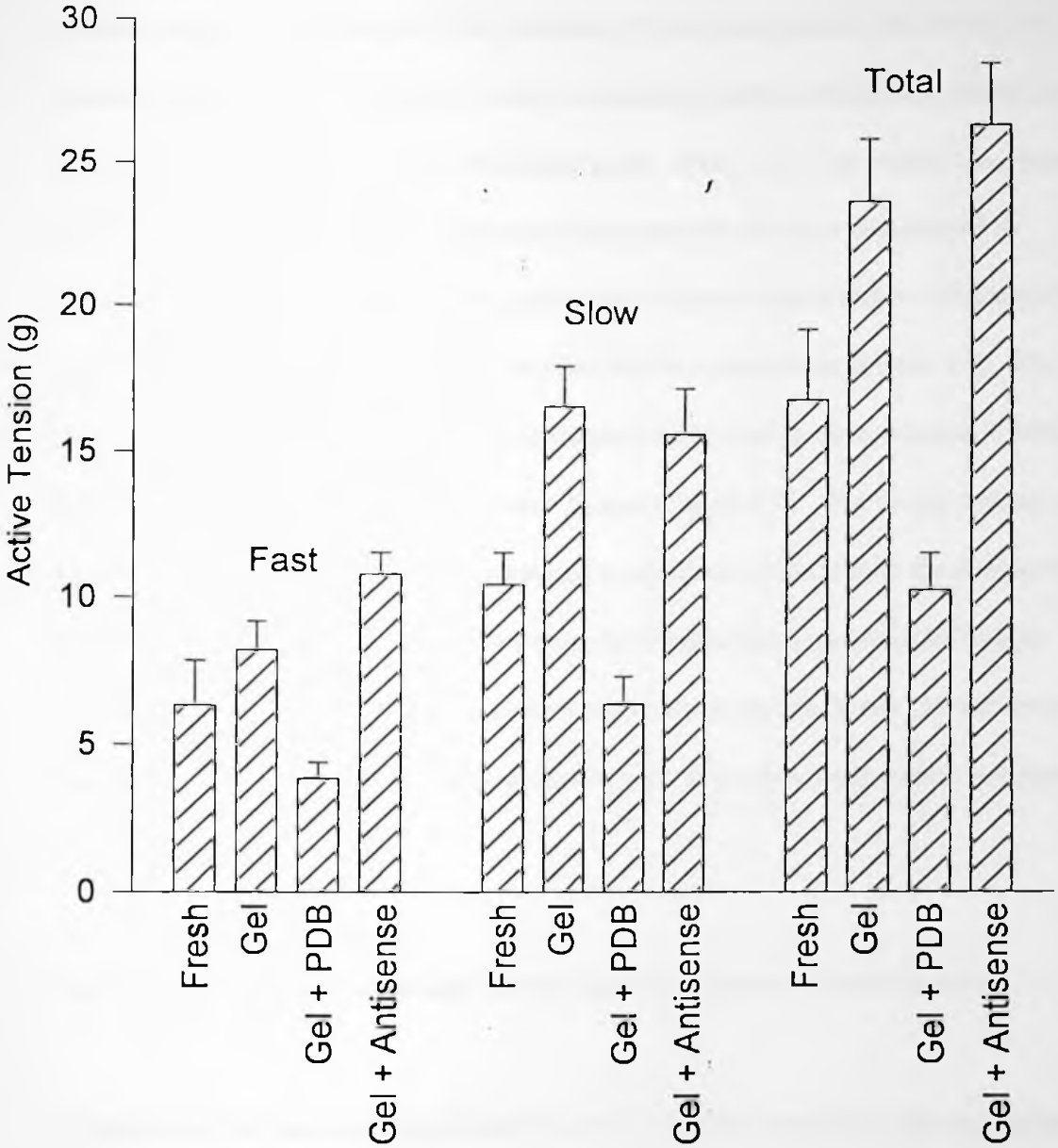

Figure 15 Effect of *in vivo* application of pluronic gel F127 on high potassium-induced contraction of rat aorta. Gel was applied alone or in combination with either 10^{-6} M phorbol 12,13 dibutyrate or kinesin antisense oligonucleotide (8 µmoles). Results are expressed as grams of active tension developed and are divided into fast and slow components of contraction. Bars represent averaged values of 6-12 tissues. * indicates a significant difference ($p < 0.05$) from fresh tissue levels.

could be used to both transport DNA into the cell and also to allow this DNA to be translated into a protein product, a vector expressing β-galactosidase was added to the gelatin matrix and applied to the abdominal aorta. After a 24 hour incubation, tissues showed a significant increase in β-galactosidase protein levels as determined by colorimetric assay (Figure 16). The application of gelatin alone had no effects on the plasma cholesterol (data not shown), nor did it affect contraction (Figure 17). The addition of PDB (10^{-6} M)to the gelatin produced reductions in the contraction, although not as markedly as in the pluronic treated tissues (Figure 17) . The incorporation of kinesin antisense oligonucleotides produced a significant reduction in the contraction (Figure 17). The presence of kinesin heavy chain antisense oligonucleotides also produced a significant reduction in kinesin heavy chain protein levels (35-40%) that was comparable to the reductions in kinesin levels seen in *in vitro* studies (data not shown).

The Effects of Kinase Inhibitors on Smooth Muscle Contraction

A prominent role has been established for several kinases in smooth muscle contraction. These include MLCK, CaMK, PKC and, most recently, MAP kinase (Figure 18). In order to directly assess the role of these kinases as well as several other kinases which are implicated in playing a role in contraction, specific inhibitors for each kinase were employed, and their effects on tissue contractility were observed. Protein kinase A (PKA) is an enzyme found in many cell types, including smooth muscle; however, it has

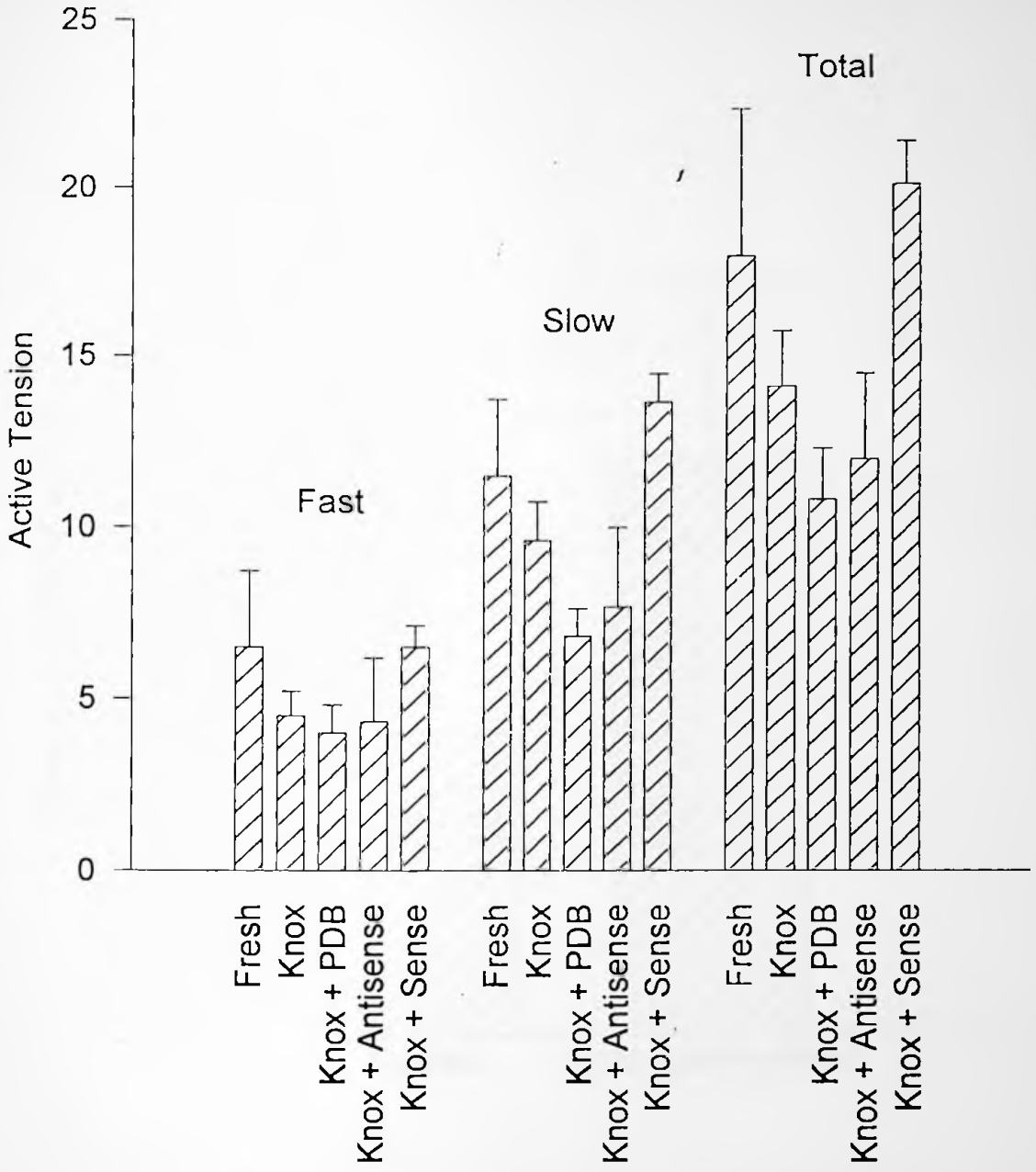

Figure 16 Effect of *in vivo* application of knox gelatin on high potassium-induced contraction of rat aorta. Knox preparation was applied alone or in combination with either 10^{-6} M phorbol 12,13 dibutyrate, kinesin antisense oligonucleotide or kinesin sense oligonucleotide (8 µmoles). Results are expressed as grams of active tension developed and are divided into fast and slow components of contraction. Bars represent averaged values of 4-10 tissues. * indicates a significant difference ($p < 0.05$) from appropriate control.

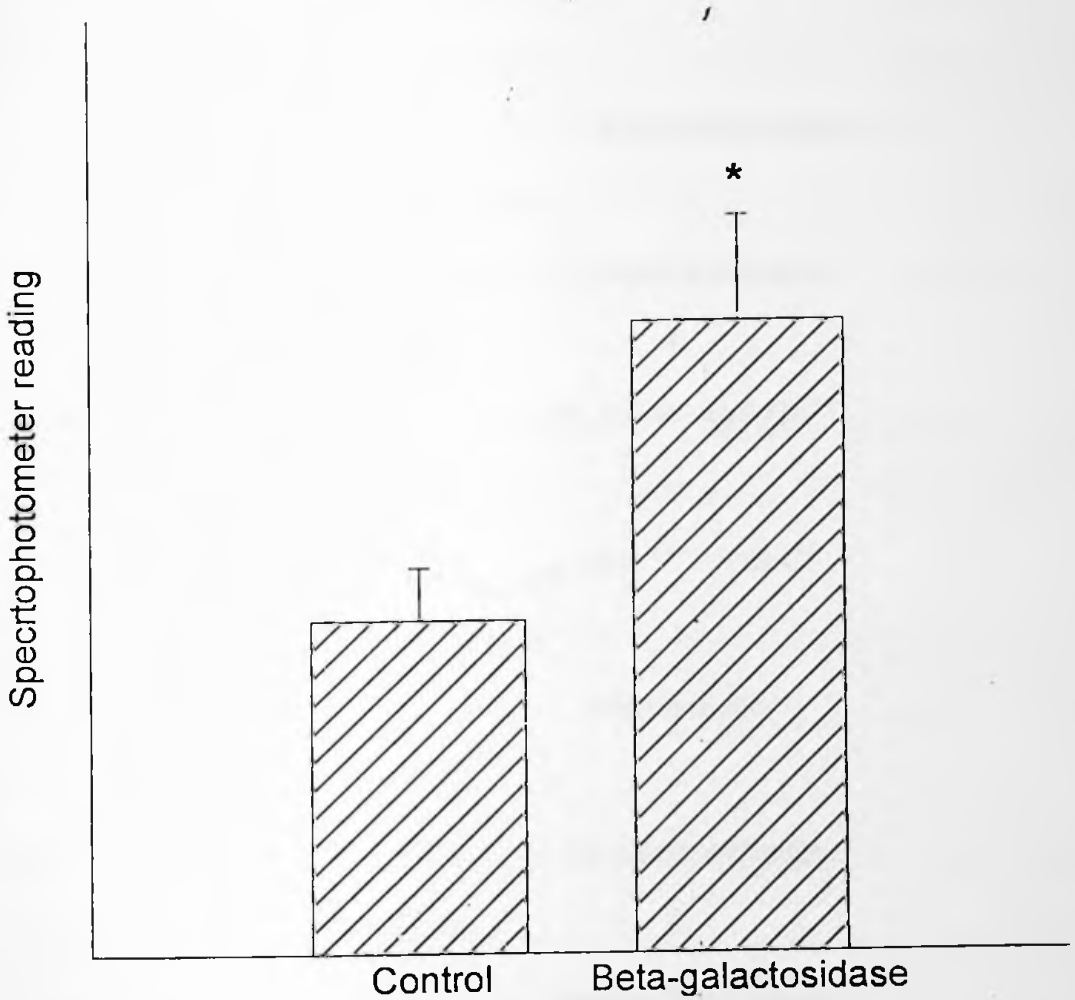

Figure 17 Efficiency of beta-galactosidase vector incorporation into rat aorta when applied in a Knox gelatin preparation. Results are expressed in arbitrary spectrophotometric units and represent averaged values of three experiments. * indicates a significant difference ($p < 0.05$) from control.

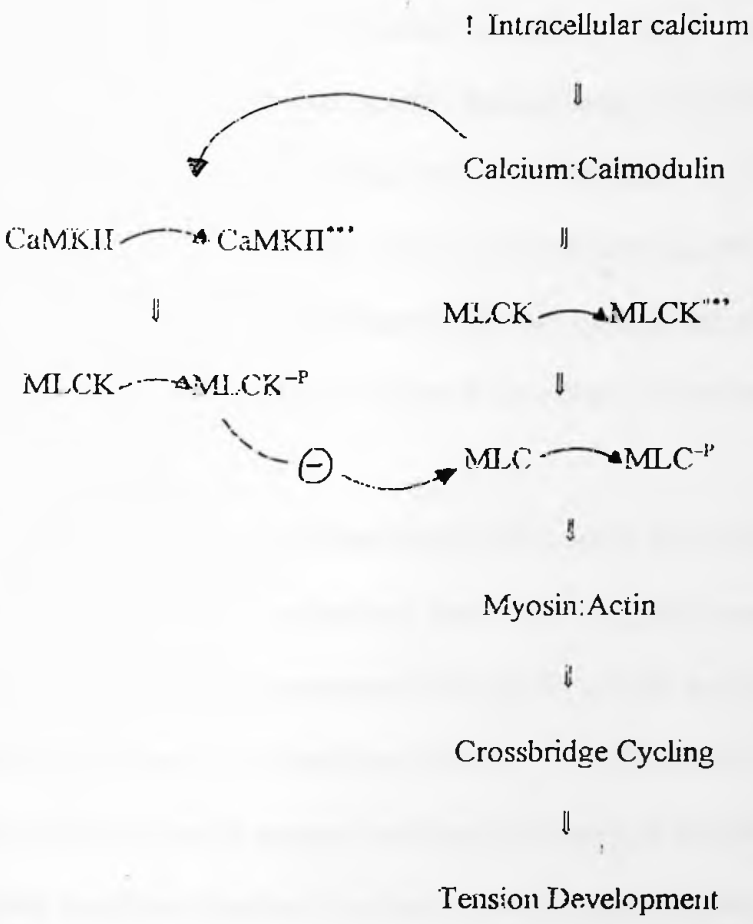

Figure 18 Diagram indicating the course of kinase phosphorylations during activation of smooth muscle contraction. MLCK, myosin light chain kinase. MLC, myosin light chain. CaMKII, calcium-dependent calmodulin kinase. --P indicates a protein phosphorylation.

not been shown have an effect on contraction. In our hands, the application of specific inhibitors to PKA had no effect on contraction (Figure 19). Tyrosine kinases are also abundant in smooth muscle, and are beginning to be suspected to play role in the contraction. In our hands, specific suppression of tyrosine kinase activity had no effects of contraction; however, when the tyrosine kinase inhibitor genistein was used at a concentration which also inhibited $pp60^{src}$, the contraction was inhibited (Figure 20). This reduction was confined to the fast phase of contraction. It is suggested in the literature that MAP kinase may play a role in smooth muscle contraction. Specific inhibition of MAP kinase by olomoucine produced a significant reduction in the total contraction, and this loss was due to a loss of fast phase contraction (Figure 21).

Of the kinases that are well established to be instrumental in producing and in maintaining smooth muscle contraction, both MLCK and PKC are suspected to facilitate or enhance smooth muscle contraction while CaMK activity is associated with a modulation of contraction. Therefore, inhibition of MLCK or PKC would be expected to decrease the level of active tension developed upon agonist stimulation, and CaMK suppression would be expected to produce either no change in the strength of contraction or to facilitate the generation of greater force by removing the repression that CaMK activity provides. In the case of PKC, the results were comparable to previously reported data; staurosporine inhibition of kinase activity caused an inhibition of contraction (Figure 22). Inhibition of MLCK with ML-9 [1- (5- chloronapthalene- 1- sulfonyl) homopiperazine HCL] also produced an inhibition of the contraction as would be

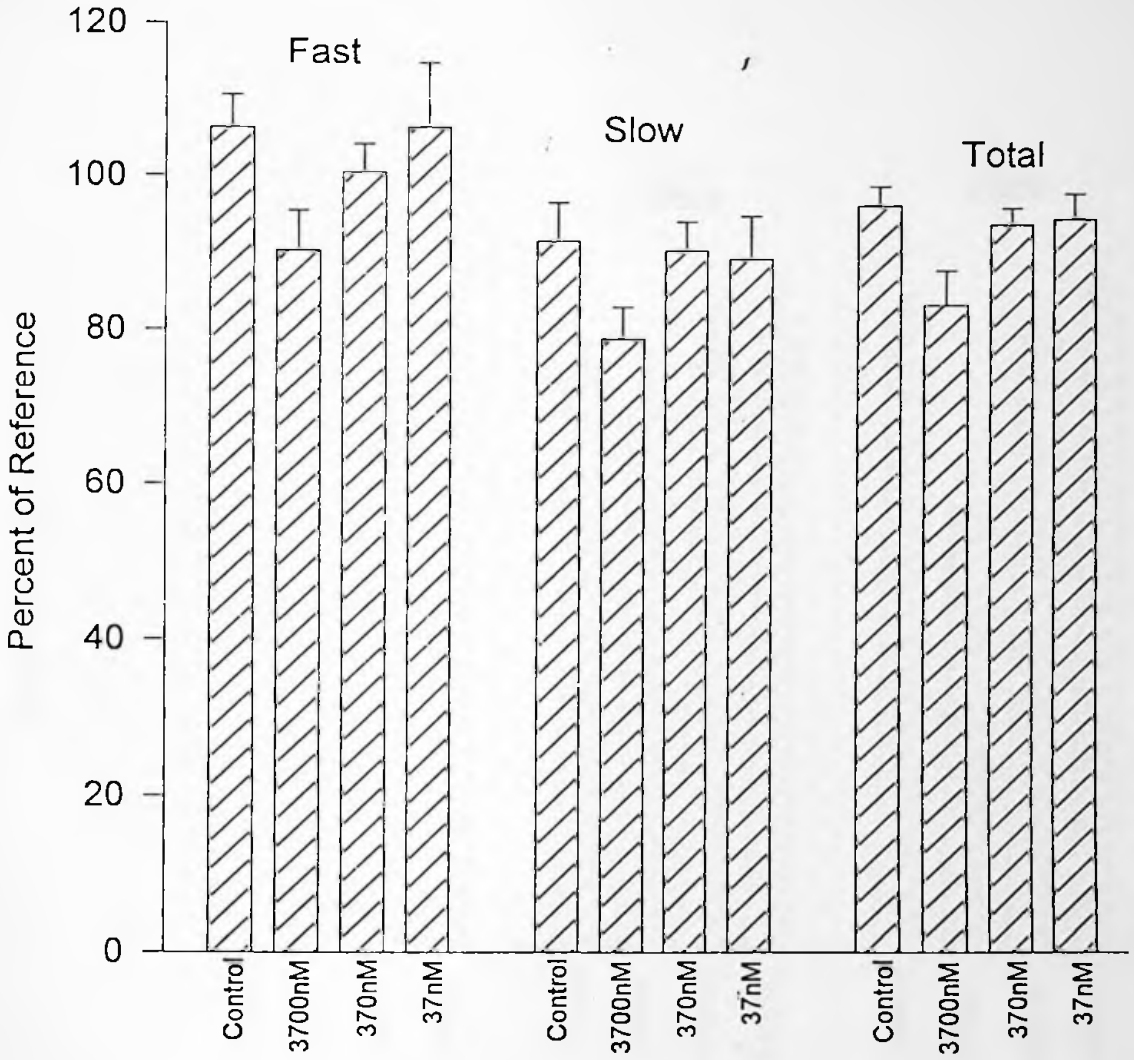

Figure 19 Effect of H-8 { N - [2 - (methylamino) ethyl] - 5 - isoquinolinesulfonamide HCL} inhibition of protein kinase A on high potassium-induced contraction in rat aorta. Values are reported as percent of reference contraction and are divided into fast and slow components. H-8 was added at concentrations of 37 nM, 370 nM or 3700 nM. Values are averages of 6-8 tissues.

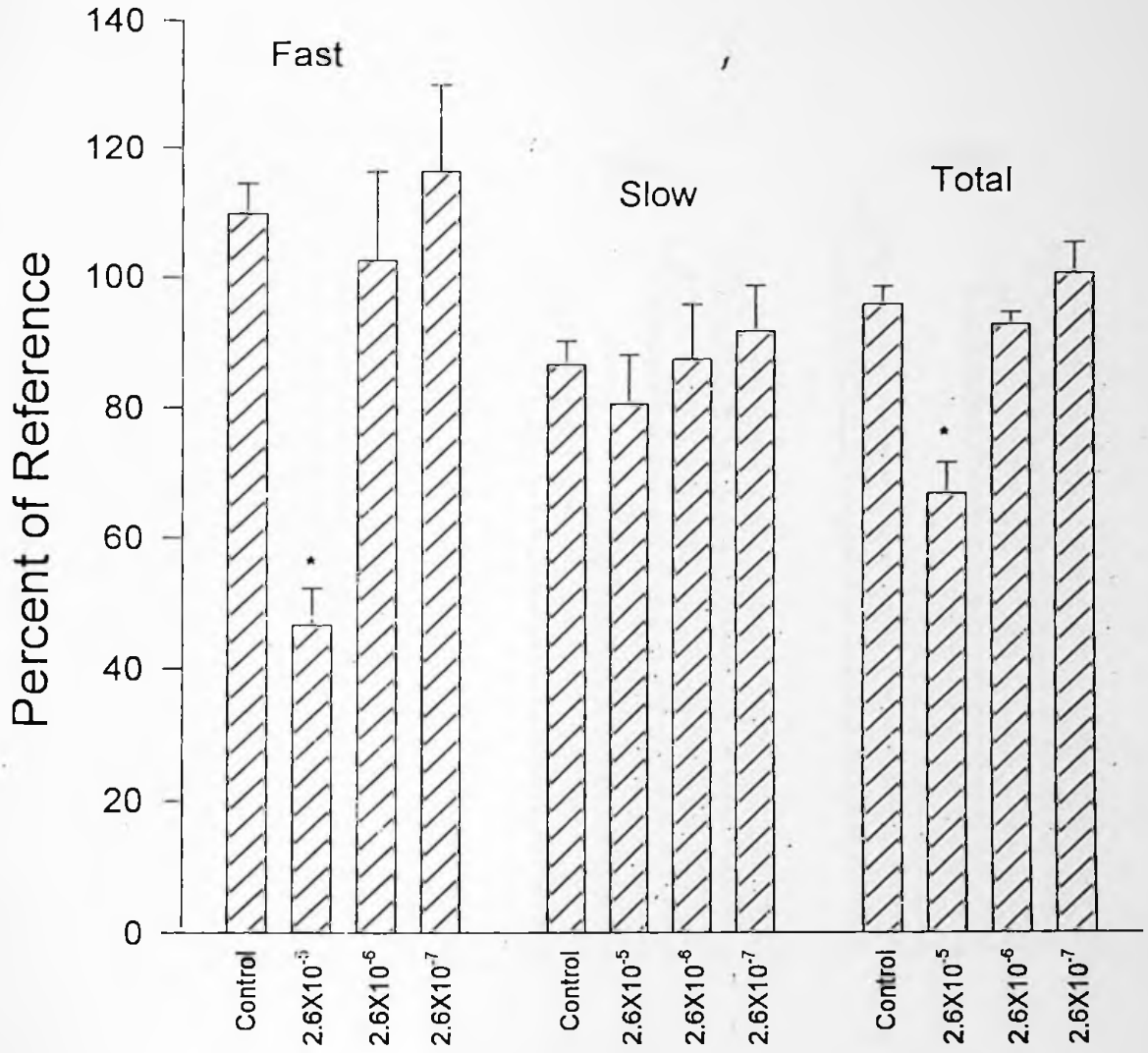

Figure 20 Effect of genistein inhibition of tyrosine kinase on high potassium-induced contraction in rat aorta. Values are reported as percent of reference contraction and are divided into fast and slow components. Genistein was added at concentrations of 2.6x 10^{-7} M, 2.6x 10^{-6} M or 2.6x 10^{-5} M. Values are averages of 6-8 tissues. * indicates a significant difference ($p < 0.05$) from control.

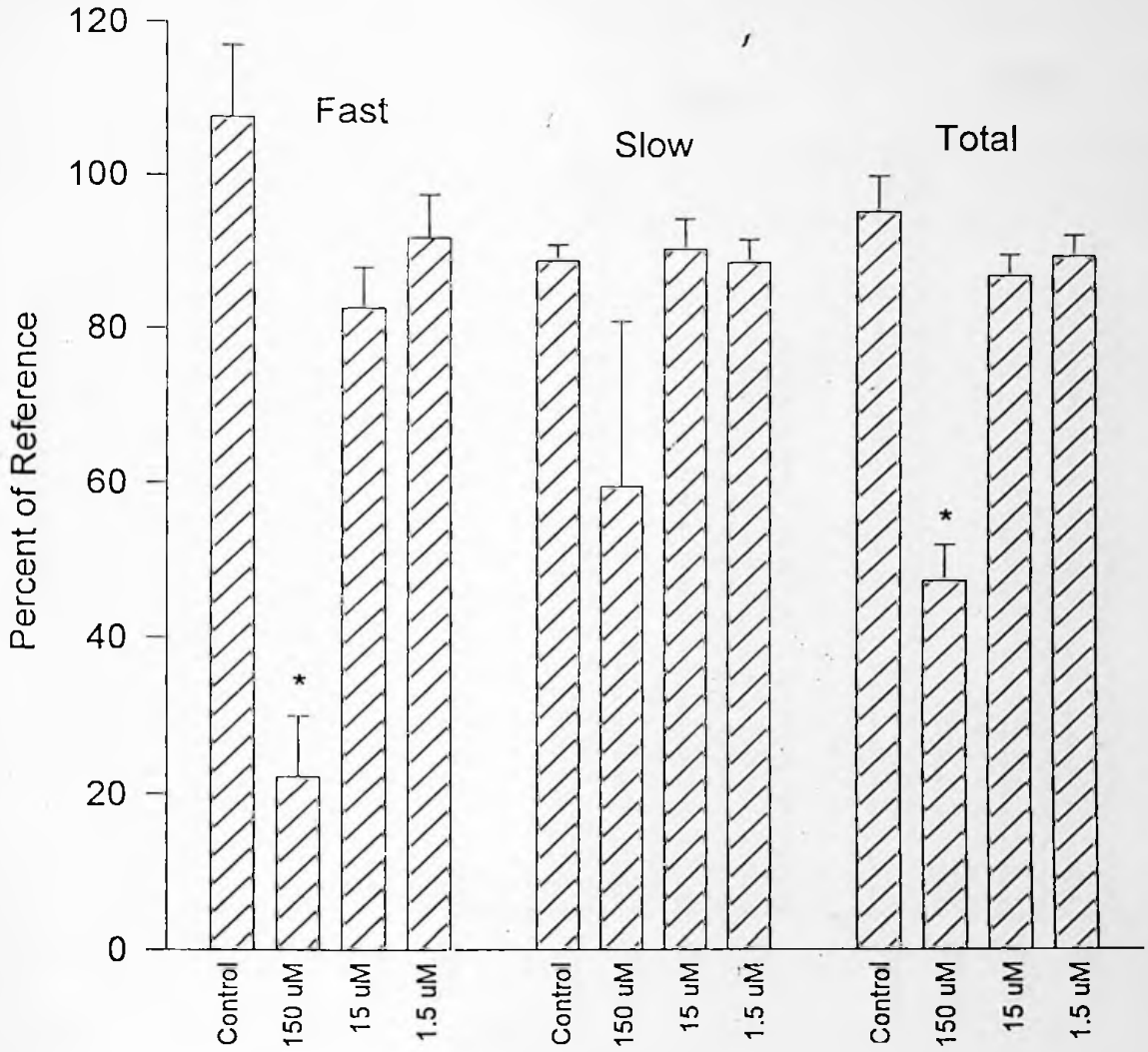

Figure 21 Effect of olomoucine inhibition of mitogen activated protein kinase on high potassium-induced contraction in rat aorta. Values are reported as percent of reference contraction and are divided into fast and slow components. Olomoucine was added at concentrations of 1.5 uM, 15 uM or 150 uM. Values are averages of 6-8 tissues. * indicates a significant difference ($p < 0.05$) from control.

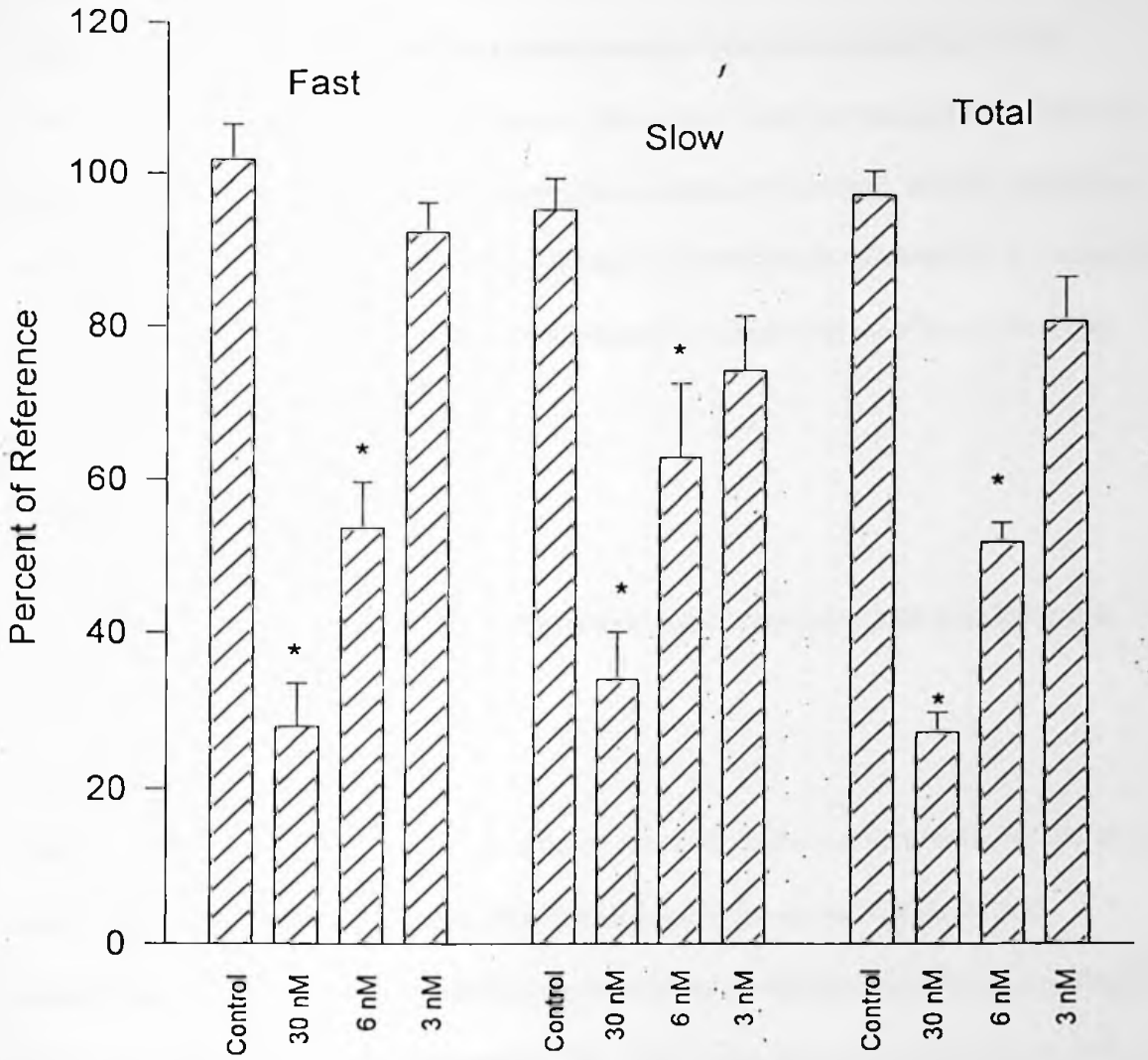

Figure 22 Effect of staurosporine inhibition of protein kinase C on high potassium-induced contraction in rat aorta. Values are reported as percent of reference contraction and are divided into fast and slow components. Staurosporine was added at concentrations of 3 nM, 6 nM or 30 nM. Values are averages of 6-8 tissues. * indicates a significant difference ($p < 0.05$) from control.

expected, however, it was found that this loss of contractility by the tissue was confined to the fast phase of contraction at a concentration corresponding to the IC_{50} (Figure 23). While this is consistent for a role for myosin phosphorylation in this portion of the contraction, it is commonly held that myosin phosphorylation is responsible for both the development and the maintenance of slow phase contraction as well. Finally, inhibition of CaMK with KN-62 {1- [N, O- *bis* (5- Isoquinolinesulfonyl)- N - methyl- L - tyrosyl]- 4- phenylpiperazine} produced a loss of contractility in both phases of the contraction (Figure 24).

Evaluation of the Interactions Between the Cytoskeleton and Protein Kinase C

Disruption of the Cytoskeleton In order to assess the effect of selective disruption of components of the cytoskeleton on PKC translocation, the drugs colchicine and cytochalasin B were utilized to inhibit polymerization of cellular microtubules and actin microfilaments, respectively (Figures 25, 26). There appears to be a substantial portion of microtubules that are quite stable and therefore, insensitive to colchicine (Dentler and Adams, 1992), but which are depolymerized by cooling to 0°C for 60 minutes (Tsutsui et al., 1993). Consequently, in most of the experiments, tissues and cells were cooled to 0°C with colchicine added just prior to rewarming to prevent repolymerization of the microtubules. Treatment with colchicine disrupted the filamentous appearance of the

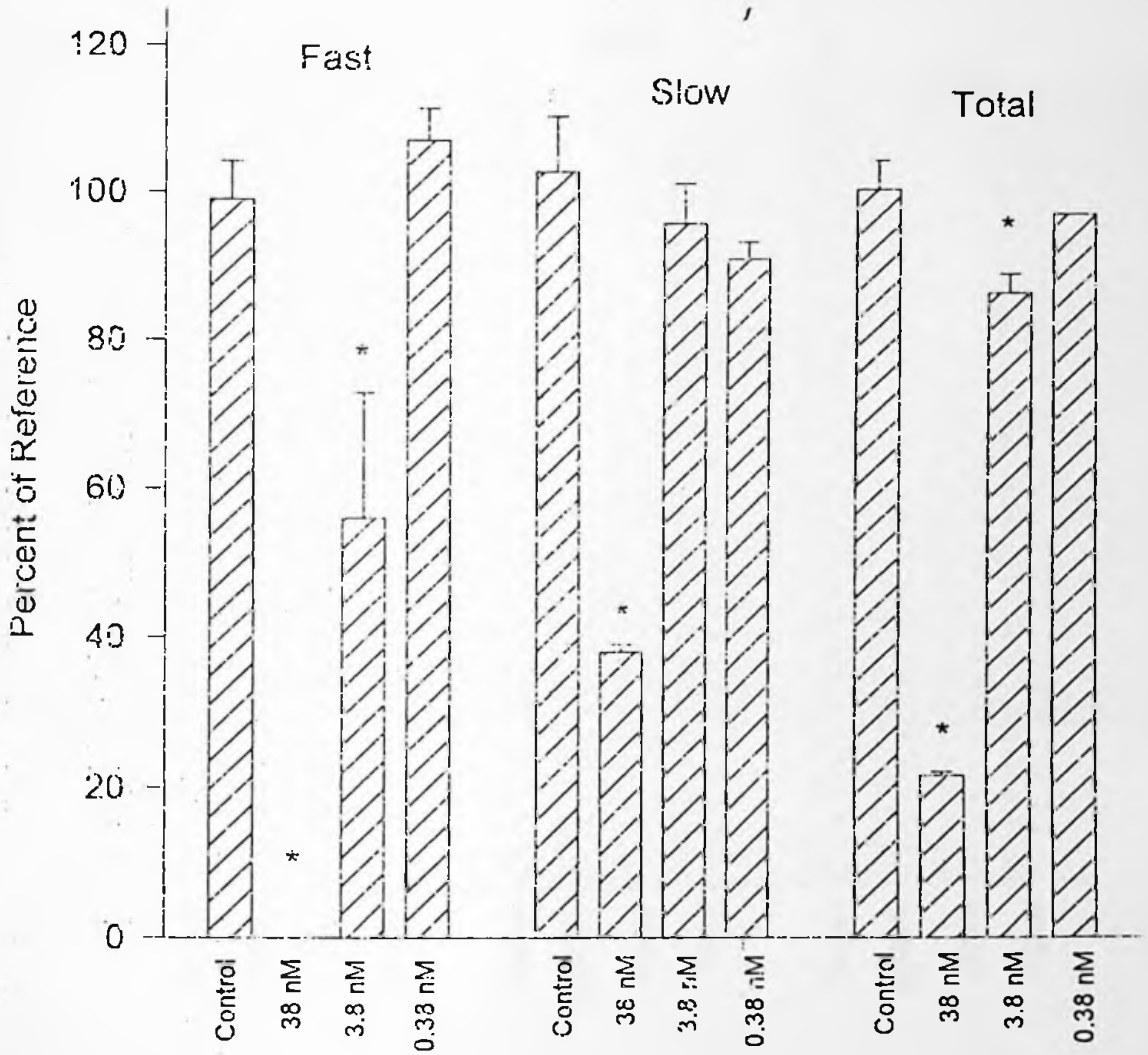

Figure 23 Effect of ML-9 [1- (5- chloronapthalene- 1- sulfonyl) homopiperazine HCL] inhibition of myosin light chain kinase on high potassium-induced contraction in rat aorta. Values are reported as percent of reference contraction and are divided into fast and slow components. ML-9 was added at concentrations of 0.38 nM, 3.8 nM or 38 nM. Values are averages of 6-8 tissues. * indicates a significant difference ($p < 0.05$) from control.

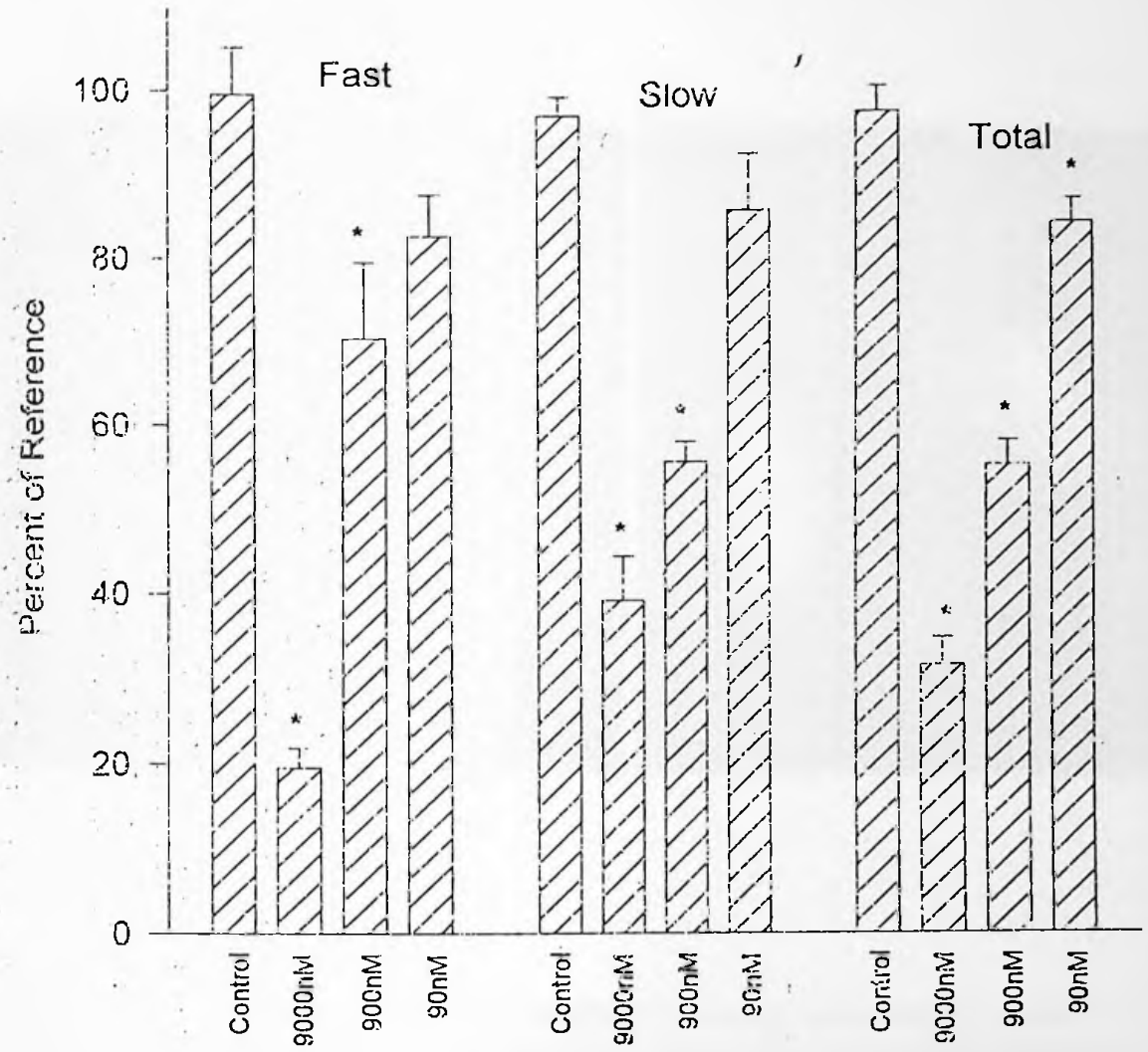

Figure 24 Effect of KN-62 {1- [N, O- *bis* (5- Isoquinolinesulfonyl)- N - methyl- L - tyrosyl]- 4- phenylpiperazine} inhibition of calcium-dependent protein kinase on high potassium-induced contraction in rat aorta. Values are reported as percent of reference contraction and are divided into fast and slow components. KN-62 was added at concentrations of 90 nM, 900 nM or 9000 nM. Values are averages of 6-8 tissues. * indicates a significant difference ($p < 0.05$) from control.

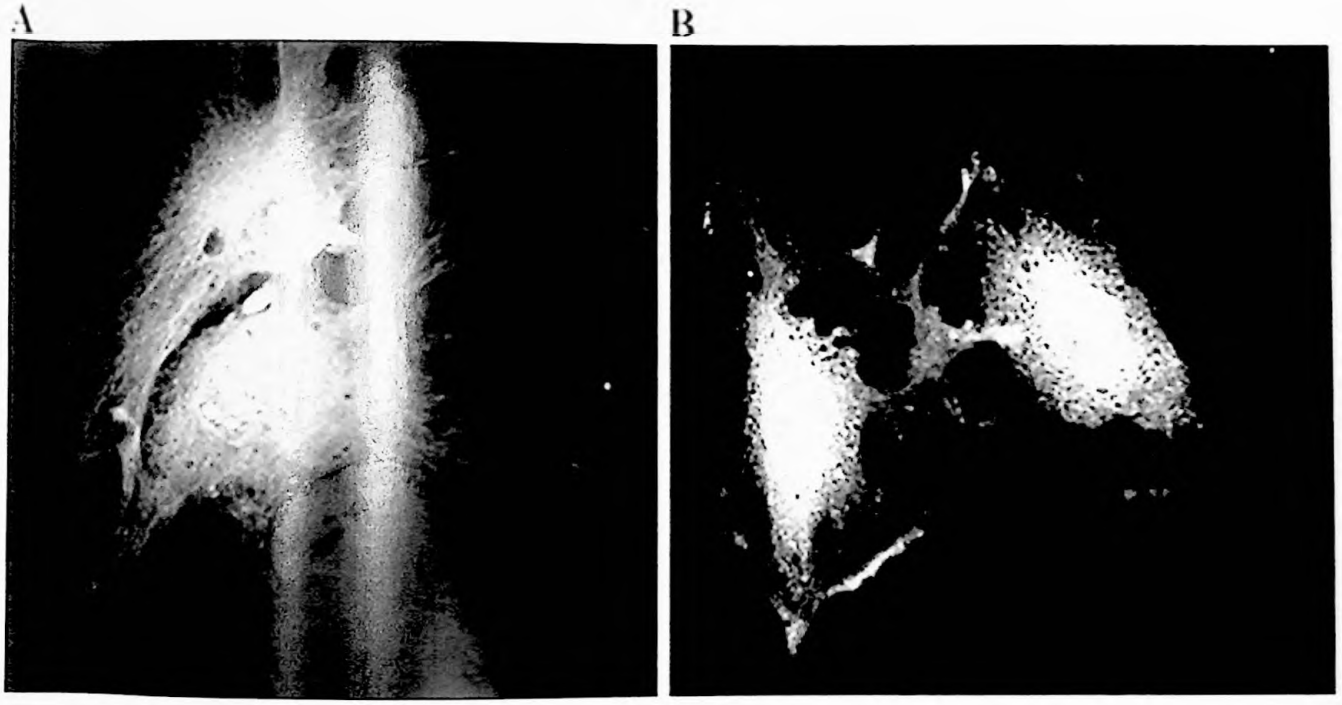

Figure 25 Confocal micrograph showing normal smooth muscle microtubular structure (a) and the effects of the microtubule disrupting drug colchicine (b). Microtubules are visualized by FITC labeled alpha-tubulin antibody.

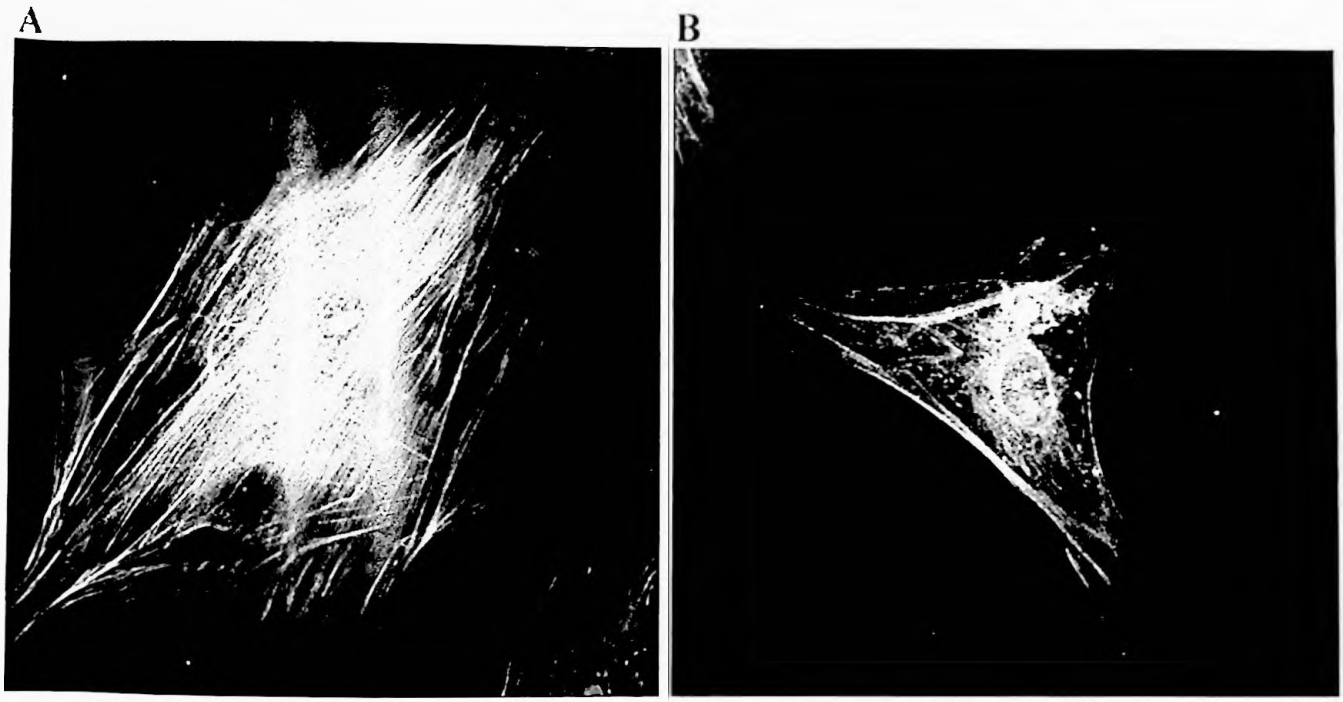

Figure 26 Confocal micrograph showing normal smooth muscle actin distribution (a) and the disruption of this portion of the cytoskeleton by the addition of cytochalasin B (b), a potent inhibitor of actin polymerization. Actin filaments are stained by the addition of Cy3 labeled phalloidin.

microtubules as observed by confocal microscopy, producing a punctate pattern of staining (Figure 25). There was evidence of some residual tubular structure remaining following colchicine treatment which is consistent with the presence of a colchicine insensitive population of microtubules (Dentler and Adams, 1992). The combination of cold treatment and colchicine produced a more extreme disruption of the microtubular network, however, a small degree of filamentous structure could still be observed in the cells (data not shown).

Treatment with cytochalasin B to disrupt the actin containing cytoskeleton produced a marked reduction in the number of actin filaments (Figure 26). However, there was not a complete dissolution of the actin cytoskeleton. This finding suggests a population of stable actin filaments that are insensitive to the actions of cytochalasin, which acts only on actively polymerizing actin. In addition to the apparent loss of F-actin, there was also a notable change in the shape of the cell. The cell membrane was observed to lose its smooth appearance and began to develop pronounced projections. This change in cell shape is presumably due to the depolymerization of cytoskeletal F-actin.

Effect of Disruption of the Cytoskeleton on PKC Translocation To determine the effects of disruption of different components of the cytoskeleton on PKC translocation, we first examined the change in the ratio of cytosolic to particulate PKC following stimulation with PDB. Western blot analysis revealed that disruption of the microtubular but not the microfilament component of the cytoskeleton significantly reduced the ratio of

PKC in the particulate to the soluble fraction of stimulated cells, suggesting the inhibition of translocation from the cytosol to membrane structure (Figure 27). The decrease in ratio was greater in cells treated by cooling plus colchicine (~80%) compared to those treated with colchicine alone (~60%) (Figure 28). The results show equally clearly that tissue rewarmed after cooling to 0° C had no impairment of PKC translocation to the particulate fraction (Figure 28).

Because Western analysis provides only an indirect measure of PKC translocation, the effect of cytoskeletal disruption on the translocation of PKC-α was studied using laser confocal microscopic analysis. Prior to stimulation, cells stained for PKC-α showed a diffuse distribution throughout the cytoplasm (Figure 29A). By comparison, the treatment of control cells with PDB to activate PKC initiated translocation resulting in intense immunostaining for PKC-α at the perinuclear region of the cell by 10 minutes after the addition of PDB (Figure 29B). Incubation of unstimulated (Figure 29C) and stimulated (Figure 29D) cells with secondary antibody in the absence of primary antibody indicated that PKC-α translocation observations were not significantly influenced by non-specific staining. As seen in Figure 30, dual immunostaining indicated that, prior to stimulation, PKC-α was diffusely distributed throughout the cytoplasm showing little or no relationship with microtubular structure. Within 30 seconds following PDB addition, PKC-α was observed to localize to filamentous strands (Figure 31B), reflecting co-localization with microtubules (Figure 31A). Although some evidence of PKC-α association with the microtubules remained at 10 minutes after PDB addition, co-

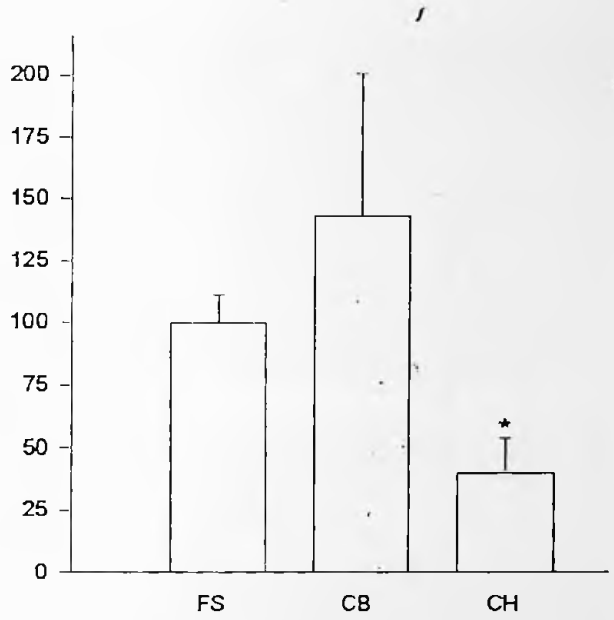

Figure 27 Bar graph showing the changes in membranous to cytoplasmic ratio of PKC-α in cultured rat aortic smooth muscle cells after treatment with cytochalasin B (CB) to inhibit actin polymerization, and colchicine (CH) to disrupt the microtubular cytoskeleton. FS denotes stimulated control tissues. Data were derived using densitometric data from Western blots and is presented as mean ± SEM. Values indicate the average from 5-7 experiments. * indicates a significant difference ($p < 0.05$) from control.

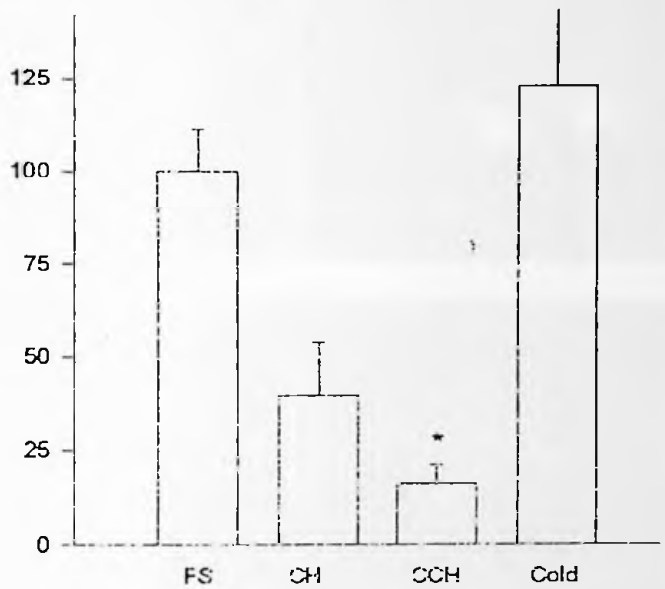

Figure 28 Bar graph showing the changes in cytoplasmic to membranous ratio of PKC-α in cultured rat aortic smooth muscle cells after treatment with colchicine only or with the combination of cold treatment and colchicine to disrupt microtubules. Data were derived using densitometric data from Western blots and is presented as mean ± SEM. Values indicate the average of 4-7 experiments. * indicates a significant difference ($p < 0.05$) from control.

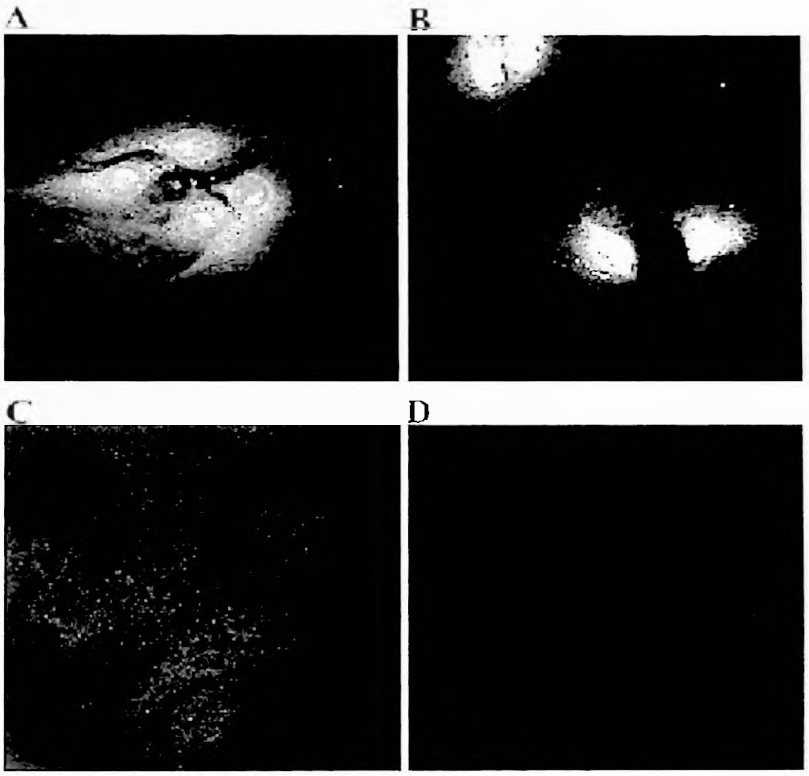

Figure 29 Confocal micrograph showing the translocation of PKC-alpha to the perinuclear region following PDB stimulation. (a) Smooth muscle cell labeled for PKC-alpha prior to stimulation exhibits a diffuse cytoplasmic localization for PKC-alpha. (b) Smooth muscle cell labeled for PKC-alpha 10 minutes after PDB addition, and PKC-alpha translocation to the perinuclear area. PKC is visualized by the addition of an unlabeled primary antibody to PKC-alpha (UBI) followed by a FITC labeled anti-mouse IgG secondary antibody. (c) Smooth muscle cells incubated with secondary antibody in the absence of primary antibody. (d) Smooth muscle cells stimulated with PDB and incubated with secondary antibody in the absence of primary antibody. Non-specific immunofluorescence is minimal in both cases and does not show any movement upon PDB stimulation, indicating that the PKC staining and translocation noted in (a) and (b) are not due to non-specific binding of the secondary antibody. Micrographs are representative from images collected in 4-9 individual experiments.

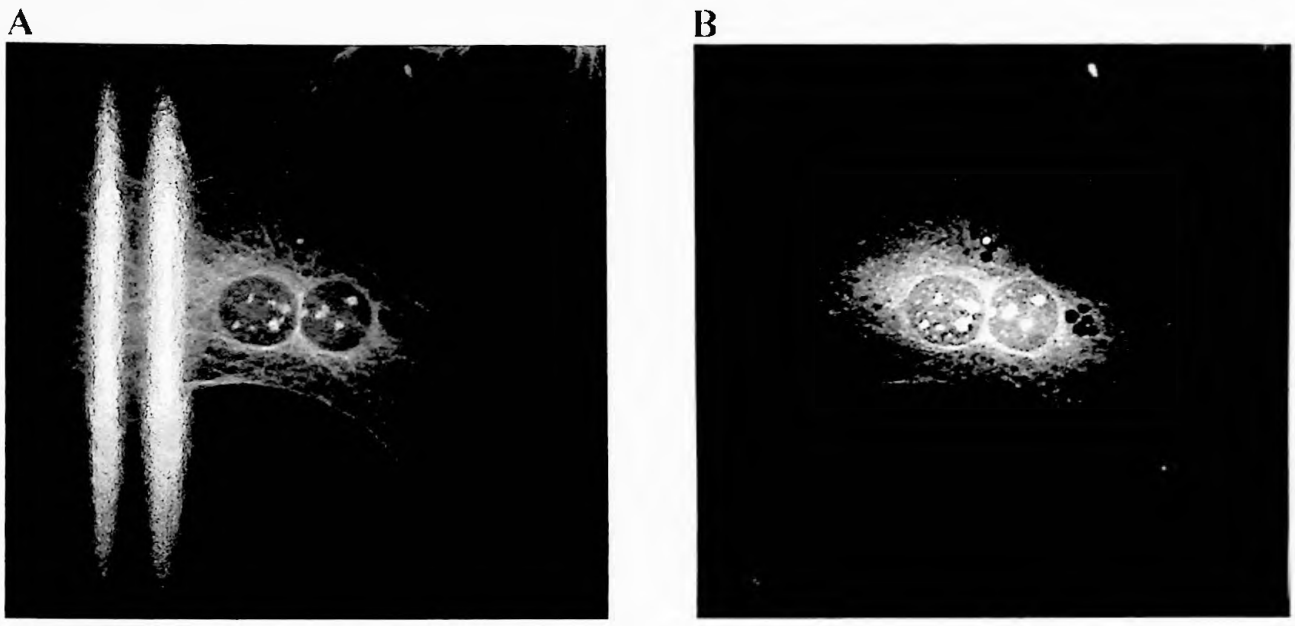

Figure 30 Dual immunostaining for the microtubules (a) and PKC-alpha (b) in unstimulated cells. PKC is visualized by Texas Red labeled secondary antibody and microtubules are stained with FITC labeled alpha-tubulin antibody. Images were collected in separate channels to prevent overlap of fluorophores. Micrographs are representative from images collected in 5 individual experiments.

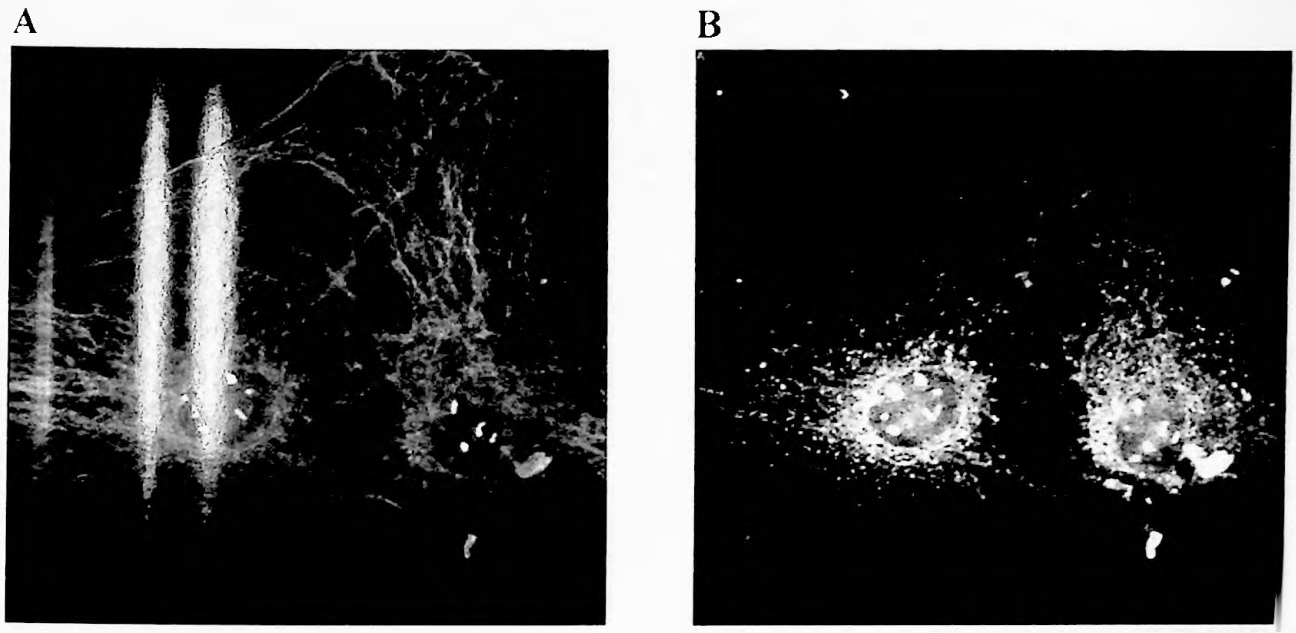

Figure 31 Dual immunostaining for the microtubules (a) and PKC-alpha (b) in PDB stimulated cells 30 seconds following PDB addition. PKC is visualized by Texas Red labeled secondary antibody and microtubules are stained with FITC labeled alpha-tubulin antibody. Images were collected in separate channels to prevent overlap of fluorophores. Micrographs are representative from images collected in 3 individual experiments.

localization of PKC-α and microtubular structure was largely obscured by PKC-α translocation and the resulting intense staining of the perinuclear region of the cell (Figure 32). Treatment with colchicine had no observable effect on the diffuse distribution of PKC-α in unstimulated cells (Figure 33a) . However, treatment with colchicine caused a dramatic inhibition of PKC-α translocation to the perinuclear membrane in PDB stimulated cells (Figure 33b). In marked contrast to the effect of colchicine, treatment with cytochalasin B at concentrations shown to severely reduce filamentous actin structure within the cell had no significant effect on PKC-α translocation (Figure 34). The results indicate that PKC-α associates with the microtubular cytoskeleton and that the directional movement of this isoform during stimulation-induced translocation requires an intact microtubular system.

Tissue Contractile Response Because PKC inhibition has been reported to specifically block slow tension development in smooth muscle (Wright and Hurn, 1994), we examined the effect of disruption of the microtubular cytoskeleton on contractile properties of the rat aortic ring as a physiological measure of PKC activation. The stimulation of smooth muscle by increasing the concentration of extracellular potassium induces contraction due to plasma membrane depolarization (Holman, 1958), resulting in the voltage-dependent activation of calcium channels (Bosca and Moran, 1993) and influx of calcium into the cell (Briggs, 1962; Urakula and Holland, 1964). The consequent increase in intracellular calcium then initiates the contractile response. The contractile response of rat aortic smooth muscle may be divided into two distinct

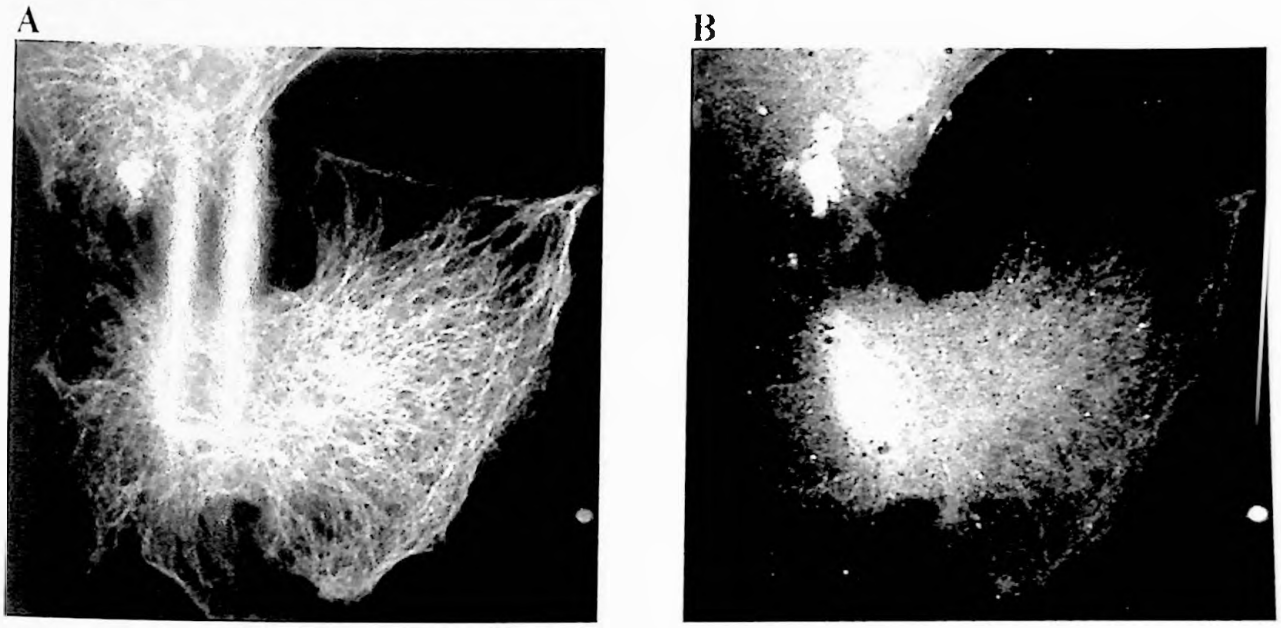

Figure 32 Dual immunostaining for the microtubules (a) and PKC-alpha (b) in PDB stimulated cells 10 minutes following PDB addition. PKC is visualized by Texas Red labeled secondary antibody and microtubules are stained with FITC labeled alpha-tubulin antibody. Images were collected in separate channels to prevent overlap of fluorophores. Micrographs are representative from images collected in 5 individual experiments.

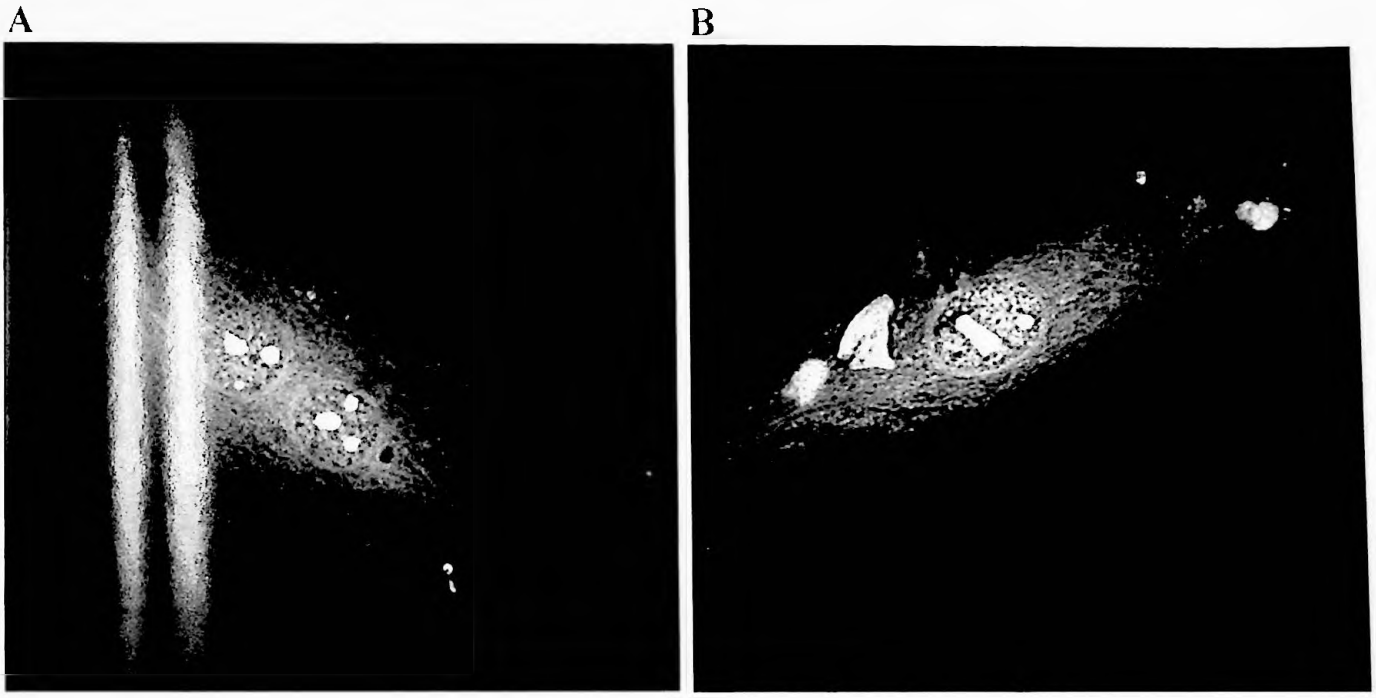

Figure 33 Confocal micrograph showing the effect of colchicine on PKC-alpha translocation. (a) Colchicine treated smooth muscle cell labeled for PKC-alpha prior to stimulation displays a diffuse cytoplasmic localization for PKC-alpha. (b) Colchicine treated smooth muscle cell labeled for PKC-alpha after PDB stimulation, movement of PKC-alpha to the perinuclear region is effectively inhibited. Localization of PKC is visualized by the addition of an FITC labeled anti-mouse IgG secondary antibody. Micrographs are representative of images collected in 4 separate experiments.

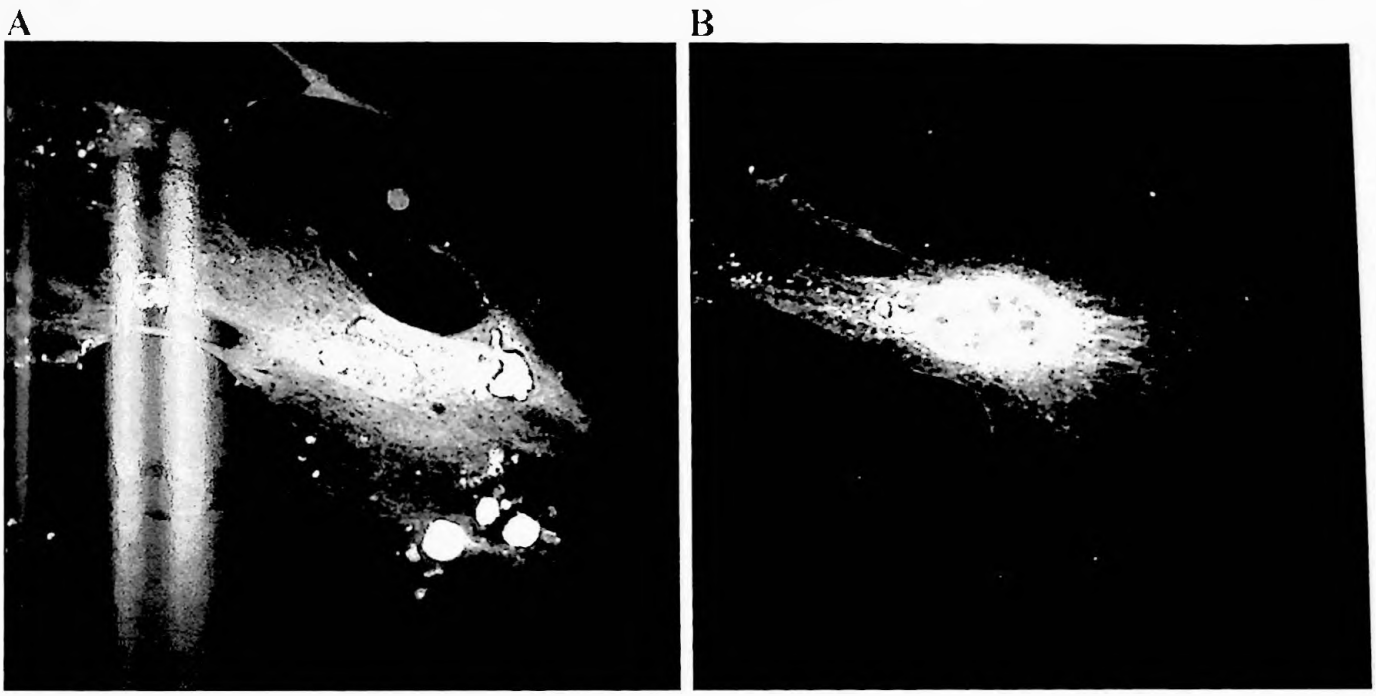

Figure 34 Confocal micrograph showing the effect of cytochalasin B on PKC-alpha translocation. (a) Cytochalasin B treated smooth muscle cell labeled for PKC-alpha prior to stimulation displays a diffuse cytoplasmic localization for PKC-alpha. (b) Cytochalasin B treated smooth muscle cell labeled for PKC-alpha after PDB stimulation. PKC-alpha is markedly translocated to the perinuclear area. PKC is stained by the addition of an FITC labeled anti-mouse IgG secondary antibody. Micrographs are representative of images collected in 4 separate experiments.

components, an initial rapid increase in tension followed by a prolonged, slow elevation of tension (Wright and Hurn, 1994). The fast component accounts for about 35% of the total tension developed and is complete within 10 seconds; whereas, the slow component requires an average of 27 minutes for completion. The fast and slow components have been shown to exhibit distinctly different physiological and pharmacological properties (Wright and Hurn, 1994) suggesting that different mechanisms regulate these components. In particular, it has been shown that very low concentrations ($8X10^{-9}M$) of the PKC antagonist staurosporine selectively inhibits the slow component of response.

Partial disruption of the microtubules by colchicine did not significantly affect the contractile response to potassium depolarization (Figure 9). By comparison, the more extensive depletion of the microtubules by the combined treatment of tissue cooling plus colchicine significantly decreased tension development with the effect confined exclusively to the slow component (~60%). The results are consistent with a direct role of PKC in slow tension development and the blockade of PKC translocation/activation by disruption of the microtubular cytoskeleton. However, the alteration of the contractile response was found to correlate only poorly with the degree of inhibition of translocation; both colchicine alone and colchicine in combination with cell cooling significantly attenuated the translocation of PKC-α while only the more severe treatment reduced the slow component of the contractile response. A possible explanation is that the colchicine treatment was less effective in tissues than in the cells in culture and that all parts of the tissue did not receive optimal exposure to the drug. It is also possible that the activation

of only a portion of the PKC available is sufficient to support slow tension increases and the more severe disruption of microtubules was required for a threshold effect on contractile responsiveness.

IV Discussion

Smooth muscle exhibits a number of unique contractile properties for which the underlying mechanisms are unknown or only poorly understood. For example, various types of smooth muscle have the ability to slowly contract and to then maintain a stable active tension for hours *in vitro*. The contractile response of rat aorta to potassium describes an initial fast phase in which approximately 35% of total force is developed within ten seconds (Wright and Hurn, 1994) while the remaining 65% of maximal force is subsequently generated over an interval of up to thirty minutes. It has been demonstrated that the contractile response of rat aortic rings can be separated into its fast and slow components on the basis of several physiological and pharmacological criteria. For example, the slow response shows markedly increased sensitivity to extracellular Ca^{2+}, temperature, and potassium depolarization compared to the fast component. In addition, it was shown that cytochalasins, which influence actin polymerization and inhibit filament elongation, could be utilized to selectively inhibit the slow response, indicating that this component requires a restructuring of the actin cytoskeleton for effective generation of force. These observations suggest that distinctly different mechanisms regulate the fast and slow components of the contractile response in aortic smooth muscle and that remodeling in the actin-containing portion of the cytomatrix is a key element in producing the slow response.

The active tension-length curve of smooth muscle is generally similar to that of skeletal muscle (Herlihy and Murphy, 1973) indicating that force is generated by a sliding filament mechanism. However, the active tension-length relationship of smooth muscle is much broader than reported for skeletal muscle (Rudel and Taylor, 1971) with the capability for contraction at a smaller fraction of L_o – defined as the tissue length which produces optimal contraction (Herlihy and Murphy, 1973; Speden, 1960) and maximal or near maximal force obtained through a relatively wide range of tissue preload and length. Furthermore, it has been shown that increasing the passive tension-preload of rat aortic rings from 1 g to 8 g increased the magnitude of the fast response but did not alter the slow response (Wright and Hurn, 1994). Hence, the broad and relatively shallow active tension preload curve of this tissue can be explained in terms of the impervious nature of slow force development to preload and tissue stretch. In light of the selective effect of cytochalasin on the slow response (Figure 8), it can be surmised that the apparent detachment of this response from the influence of preload is due to the formation or rearrangement of a portion of the tension bearing structure associated with the contractile protein following stimulation of the tissue.

The present results (Figure 8) confirm earlier findings that the application of cytochalasin prior to tissue stimulation selectively blocks the slow response without changing the passive tension-length relationship of the tissue (Figure 5). Cytochalasins do not influence force development in skeletal (Alberts et al., 1983) or cardiac (Tsutsui et al., 1993) muscle and are ineffective in the disassembly of the highly structured and stable

actin/myosin assemblies found in sliding filament arrangements (Croop and Holtzer, 1975). In addition, recent work has ruled out the possibility that cytochalasin causes its effects in smooth muscle via alterations in calcium influx, intracellular calcium, membrane potential or myosin light chain phosphorylation which are unchanged with cytochalasin treatment (Obara and Yabu, 1994). Hence, the effects of cytochalasin on smooth muscle appear to be mediated entirely through its inhibitory effect on actin polymerization. Because the fast component of the contractile response of aortic smooth muscle is not altered by cytochalasin (Figure 8), it may be concluded that this component involves a stable actin filament structure. Because cytochalasins can be utilized to block the slow response if applied prior to tissue stimulation (Figure 8) but have little effect on tension if they are introduced into the bath after tension development has plateaued (Wright and Hurn, 1994). These results suggest that actin polymerization and filament elongation are critical elements of slow force development and that the actin filaments created are stable against cytochalasin attack upon completion of the slow response.

In order to explain the broad tissue length-active tension curve and the relative isolation of the slow component of the contractile response from the effect of increasing tissue length, a restructuring of the cytoskeleton can be proposed that would provide a mechanism for the internal adjustment of tension on the contractile apparatus. This theoretical system for tension adjustment occurs in conjunction with the contractile proteins, not as a stand-alone structure. Myosin-associated actin is found in rosettes or cross-linked bundles (Cooke and Fay, 1972) that provide for multiple filament

attachments extending to anchorage points at the membrane or at other sites in the cell. Stretch results in a less than optimal association between the intercalating actin and myosin filaments and adjustment to allow the actin filament to move into a more optimal position for interaction with myosin could occur by three possible mechanisms. Actin filaments could disengage and reattach to other anchorage sites, slippage at the anchorage site could lessen tension on the actin filament, or polymerization of actin filaments from the bundle to new anchorage sites with disassembly of filaments under tension could realign the interacting actin-myosin complex. Based on the dramatic effect of cytochalasin inhibition of actin polymerization on the contractile response, new filament formation is probably the most likely mechanism by which the cytoskeleton is restructured for adjustment of tension and regulation of the spatial relationship of actin and myosin filaments.

The linking of the concept of an adjustable cytoskeletal tension-bearing element with the assumption that there is only partial or asynchronous activation of the cell complement of contractile protein during the interval following agonist stimulation in which $[Ca^{2-}]_i$ declines or cycles, could further explain slow tension increase and the long-term maintenance of tension in smooth muscle. In the interval immediately following agonist stimulation and the elevation of $[Ca^{2+}]_i$, the full complement of contractile protein would contract resulting in a relatively abrupt development of force comparable to the fast component of response. Subsequently, during the interval of declining $[Ca^{2+}]_i$ and decreased myosin light chain phosphorylation (Zhang and Moreland, 1994), only a

portion of the contractile protein would be activated at any given time, effectively resulting in asynchronous activation/inactivation of the contractile protein. An accompanying remodeling of tension-bearing actin cables would serve to lock the system at the existing tension and preserve increases in force generated by the activated complement of contractile protein. Hence, the system would act as a ratcheting mechanism with the rate of increase in force primarily determined by the percentage of contractile protein activated at any given time. The plateau would correspond to the interval of very low level activation of the contractile protein with tension maintained by the restructured tension-bearing components of the system.

In addition to indicating a role for actin filaments, the results suggest that cellular microtubules could contribute to the contractile properties of smooth muscle. The treatment of aortic rings with colchicine at 37°C enhanced the fast response, shifted the active tension-passive tension preload curve of the slow response to the left, and caused the passive tension preload-length curve to shift to the right. The last observation indicates that the system of microtubules contributes to the mechanical properties of the tissue and that partial disassembly of this system results in increased resistance to stretch by the passive tension preload. Because of the high flexural rigidity and expected inextensibility of microtubules (Gittes et al., 1993), the microtubular system would not be expected to convey increased elasticity to the tissue. Hence, it may be reasonable to speculate that the microtubules provide a support structure for elements directly responsible for the lowered resistance to stretch in control tissues compared to colchicine-

treated tissues. It has been shown that the structural arrangement of intermediate filaments is altered following microtubule disassembly by colchicine alkaloids in fibroblasts (Virtanen et al., 1980) suggesting that this treatment has secondary effects in the cytoskeleton.

The effects of colchicine at 37°C on active tension were quite different in the fast and slow components of the response. The mechanism of enhancement of the fast response by microtubule dissolution (Figure 9) is not clear, but may be similar to that operating in pressure hypertrophied myocardial cells exhibiting increased contractile responsiveness following treatment with colchicine or lowered temperature to cause microtubule disassembly (Tsutsui, et al., 1994). These studies indicated that the effect of colchicine to enhance contraction was specific to the microtubules and suggested that the microtubule component of the cytoskeleton acted to create a viscous load that would impede sarcomere shortening. By comparison, disruption of microtubules attenuated the slow response at preloads above 5 grams, effectively shifting the active tension-length curve to the left for this component. This strongly suggests that the microtubules play a role in determining the mechanical properties of tissue specifically with regard to slow force development.

Microtubules play a central role in numerous force-generating processes by acting as the structural element for the movement of cellular motor proteins. These microtubule-associated motors are presently grouped into the kinesin, dynein, and dynamin

superfamilies. Kinesin is a heterotetramer consisting of two heavy chains and two light chains (Bloom et al., 1988; Kuznetsov et al., 1988). The heavy chains contain the highly conserved motor domain and additional sites suspected of regulating motor activity and mediating interactions between the light chains and cargo carried by the motor (Walker and Sheetz, 1993). A number of kinesin-like proteins have been reported that are similar in the motor domain but are variable in other regions suggesting these proteins will differ in the type of cargo carried (Vale and Goldstein, 1990). Moreover, recent evidence suggests that kinesins may be able to interact with more than one microtubule and kinesin- like proteins may interact with actin filaments (Hoyt, 1994). At present, the kinesins are implicated in a number of cell functions and this can be expected to greatly expand as more kinesin-related proteins are discovered and the full range of their interactions with the cytoskeleton and other components of the cell are understood.

It has been reported (Matthies et al., 1993) that extensively purified bovine brain kinesin showed several forms of heavy and light chains. Using SDS-PAGE analysis the group demonstrated at least three forms of kinesin heavy chain with molecular masses between 120 and 130 kDa and a high molecular weight species above 200 kDa which they suggested could be an aggregate containing kinesin heavy chain. To our knowledge, the kinesin profile in SDS-PAGE analysis of rat aortic smooth muscle has not been previously examined. Our results show a species of kinesin at approximately 116 kDa in fresh aortic tissue. However, in cultured tissues we see proteolytic fragments of kinesin

heavy chain indicating a high rate of degradation *in vitro*. Also, on occasion a high molecular weight species was noted that appears similar to the observation of Matthies et al. (1993) in neural cells. The proteolytic fragments appear as a triplet band at 85 kDa, a single band at 70 kDa, and a triplet band at 48 kDa. These bands are consistent with kinesin fragments seen in *Xenopus* muscle as reported by Sigma Immunochemicals. Antisense oligonucleotides have been previously used to successfully block kinesin synthesis in neural cells in culture (Matthies et al., 1993) and *in situ* (Amartunga et al., 1993) with estimated suppression of kinesin heavy chain protein levels in excess of 95% within 24 hours after addition of antisense oligonucleotides (Matthies et al., 1993). Previous work with *in situ* application of oligonucleotides has commonly utilized pluronic gels as vehicles for oligonucleotide delivery. Pluronic gels are detergent-like compounds that have the characteristic of rapidly raising both plasma cholesterol and triglyceride levels when introduced in whole animal studies (Wout et al., 1992). Because elevated plasma cholesterol levels have been shown to cause increases in smooth muscle contractile response (Yokoyama and Henry, 1979), we developed a modified method for delivery of oligonucleotide. We found that a Knox gelatin preparation could be used to selectively place kinesin antisense oligonucleotides around the abdominal aorta and, in our hands, the application of antisense oligonucleotides to aortic rings *in vitro* or to the abdominal aorta *in situ* produced a reduction of kinesin protein levels by 35%. The difference in efficiency of antisense suppression in this work and that previously reported may be due to the inherent differences between oligonucleotide delivery in the intact tissues and the dispersed cell preparation, or it may be due to differences in the tissue

types. In the present study, employing antisense oligonucleotide against the kinesin heavy chain to the abdominal aorta *in situ*, and to the thoracic aorta in organ culture produced identical results, a 35% to 40% reduction in the contractile response, compared to sense oligonucleotide-treated tissues (Figure 12). The results suggest that the kinesins could play a role in the development of force by aortic smooth muscle, either through a structural support function or through a role in the transduction process.

Several of the kinases reported to be involved in transducing agonist stimulation into smooth muscle contraction were examined with respect to their effects on the fast and slow portions of the smooth muscle contraction. MLCK is considered to be the initiating kinase of smooth muscle contraction. We found that specific inhibition of MLCK activity caused a significant, but not complete, inhibition of contraction. In addition, this inhibition was primarily in the fast component of contraction with little or no effect on the slow phase. While this finding corroborates evidence suggesting that the fast phase of contraction may be regulated and function similarly to striated muscle contractile mechanisms, it suggests that development of slow tension is not solely regulated by MLCK activity. Inhibition of calcium dependent calmodulin kinase (CaMKII) also produced unexpected results. Based on what is known about the role of this kinase in the smooth muscle contraction, we expected that its inhibition would either produce no effect or would potentiate the contraction. We found that inhibition of CaMKII produced an inhibition of tension generation and that this was in both the fast and slow portions of the contraction. This finding would indicate that there may be a secondary role for CaMKII

in the mechanism of smooth muscle contraction. This could possibly be through differential effects of CaMKII on MLCK phosphorylation, phosphorylation of caldesmon, or via an effect on mitogen activated protein (MAP) kinase activity. Although MAP kinase has also been suggested to play a role in smooth muscle contraction, the mechanism for its function is not known. Inhibition of MAP kinase produced an inhibition of fast phase contraction, possibly indicating a role for MAP kinase in the regulation of MLCK activity or actin:myosin cycling.

The role of PKC in smooth muscle contraction has been extensively examined, and it appears that PKC is involved in the generation and maintenance of contraction. This involvement seems to be primarily in the slow phase contraction, although the current work also shows some effect on fast phase contraction as well. One of the unique features of PKC is that it must be translocated within the cell in order to be fully activated. Prior to activation, PKC is found diffusely throughout the cytoplasm. Following stimulation, it translocates to cellular membranes where it becomes fully activated.

The mechanism of PKC translocation and the basis for isoform targeting and substrate specificity is only recently becoming clear (Haller et al., 1990). The work of Bosca and Moran (1993) indicated the importance of calcium and phorbol ester, a synthetic form of diacylglycerol, in the initiation of translocation through an unfolding of the PKC molecule. This conformational change uncovers hydrophobic tryptophan residues that

are concealed within the hydrophilic α-helical structure of the unactivated kinase. They concluded that the hydrophobicity and the increased affinity for membrane phospholipid of the activated molecule would prompt movement from the cytosol to membrane localization sites. Because PKC isoforms are translocated to different sites, however, this mechanism did not adequately explain the specificity of isoform translocation. The case for selective isoform binding as the mechanism underlying differential localization of isoforms following activation was greatly strengthened by the discovery of receptors for activated C kinase (RACKs) (Mochly-Rosen et al., 1991). This family of receptors is found in the membrane and contains binding sites that are specific for individual activated isoforms (Mochly-Rosen, 1995). Hence, the presence of these proteins would allow only a specific isoform of activated PKC to bind to a particular subcellular compartment once the PKC molecule came in proximity to its complimentary RACK. The intermediate step of this process, the means by which PKC is physically moved from the cytosol to the membrane sites is generally thought to be due to simple diffusion. However, in view of the potential diffusional barriers presented by the cellular cytoarchitecture, it may be reasonable to suspect that movement by diffusion would be poorly controlled and incomplete.

The present results indicate that following the activation of PKC-α by addition of PDB, this isoform was seen to extensively and rapidly co-localize with microtubules during translocation to the perinuclear membrane (Figure 31). Use of colchicine or colchicine plus cold to disrupt the microtubular system within the cell dramatically reduces the

relocation of PKC-α from the cytosol to the particulate fraction (Figures 27,28) and prevents the translocation of the molecule to the perinuclear membrane (Figure 33). These findings corroborate contractile data indicating that the microtubules play an important role in smooth muscle contraction and that this is due to their function in the transport of PKC during the translocation process. Because inhibition of kinesin protein synthesis through the application of antisense oligonucleotides resulted in a corresponding reduction in the contractile response of rat aortic smooth muscle, and because of the effects of microtubular disruption on PKC translocation, it may be reasonable to speculate that the importance of kinesin to the smooth muscle contraction could be due to kinesin-mediated transport of PKC. Translocation of PKC via this type of highly structured system could result in a more controlled and efficient PKC movement to specific subcellular sites as compared to random diffusion. Kinesin-like proteins, while maintaining a high degree of homology within their motor domains, display variability within the domains involved in cargo binding. This opens the possibility for individual motor protein types to be involved in the selective transport of different cargo molecules to specific subcellular locations. This type of regulation could be important under conditions of physiological cell stimulation where differential transport could act as a means of compartmentalizing the activity of several simultaneously acting isoforms.

In studies reported here, only translocation of the alpha isoform of PKC was examined. This isoform is present in high concentrations in cultured rat aortic smooth muscle cells

and the characteristics of its translocation to the perinuclear area have previously been described in passaged cells (Haller et al., 1990). However, of the five PKC isoforms identified in smooth muscle (Kahlil and Morgan, 1996), only evidence for the involvement of PKC-ϵ in contraction has been obtained. Upon agonist stimulation, this isoform translocates before or during the interval of contraction (Kahlil and Morgan, 1993) and has been further shown to induce a calcium-independent contraction in permeabilized ferret aortic cells (Horowitz et al., 1996). Because both the translocation of PKC-α and the contractile response of vascular smooth muscle were inhibited by disruption of the microtubules, these results suggest that microtubular transport could affect more than one PKC isoform.

V Summary

In summary, disruption of cellular microtubules or inhibition of actively polymerizing actin selectively inhibits the slow component of contraction. This suggests that both of these components of the cytoskeleton contribute to slow tension development either through a structural role or through involvement in the transduction process. Additionally, disruption of the microtubular system prevents the translocation of PKC-α to the perinuclear membrane. This, coupled with the observation that the blockade of microtubule associated motor protein synthesis attenuates the contractile response, suggests that the microtubules play an important role in smooth muscle contraction by functioning as a directional transport system for the translocation of PKC. These results further indicate that both the non-actin:myosin cytoskeleton and the array of kinases present in smooth muscle play a vital role in the generation of active contractile force. Because both of these components are potentially important in producing and regulating smooth muscle contraction, further investigation into their actions and interactions is necessary and studies to this end should prove to be greatly beneficial to our understanding of the mechanics and regulation of smooth muscle contraction.

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