

Aus dem Institut für Physiologie der Medizinischen Fakultät Charité
der Humboldt-Universität zu Berlin

DISSERTATION

**Analyse der Funktion der nichtmuskulären schweren Myosinketten
in glatten Muskelzellen**

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ABBREVIATIONS

ADP	Adenosine diphosphate
aNM-MHC	Antibody against non-muscle myosin heavy chain
aS-actin	Antibody against smooth muscle α -actin
aSM-MHC	Antibody against smooth muscle myosin heavy chain
ATP	Adenosine triphosphate
$[Ca^{2+}]_i$	Intracellular calcium concentration
CaD	Caldesmon
CaM	Calmodulin
CaP	Calponin
DAG	1,2-diacylglycerol
F-actin	Filamentous actin
G-actin	Globular actin
GDP	Guanine diphosphate
GTP	Guanine triphosphate
HMM	Heavy meromyosin
InsP ₃	Inositol 1,4,5-trisphosphate
KO	SM-MHC knockout
M ₁₁₀₋₁₃₀	Regulatory subunit of myosin light chain phosphatase
MHC	Myosin heavy chain
MLC	Myosin light chain
MLC ₁₇	Essential myosin light chain
MLC _{17a}	Acidic MLC ₁₇ isoform
MLC _{17b}	Basic MLC ₁₇ isoform
MLC ₂₀	Regulatory myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
ML/s	Muscle length per second
NMA	Non-muscle myosin heavy chain isoform with 196 kDa
NMB	Non-muscle myosin heavy chain isoform with 198/200 kDa
NM-MHC	Non-muscle myosin heavy chain

nt	Nucleotide
P _i	Inorganic phosphate
PC	Phosphatidylcholine
PDBu	Phorbol 12,13-dibutyrate
PIP ₂	Phosphatidylinositol 4,5-biphosphate
PLC	Phospholipase C
PLD	Phospholipase D
PP-1C	Catalytic subunit of myosin light chain phosphatase
PKC	Protein kinase C
Rho-GDI	Guanine nucleotide dissociation inhibitor
Rho-GEFs	Guanine nucleotide exchange factors
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SMA	Smooth muscle myosin heavy chain isoform without 5'-insert
SMB	Smooth muscle myosin heavy chain isoform with 5'-insert
SM1	Smooth muscle myosin heavy chain isoform with 204 kDa
SM2	Smooth muscle myosin heavy chain isoform with 200 kDa
SM-MHC	Smooth muscle myosin heavy chain
V _{max}	Maximal shortening velocity
WT	Wild-type

INTRODUCTION

Smooth muscle forms the contractile portion of many organs like blood vessels, uterus, stomach, intestines, urinary bladder and airways. Consequently, abnormalities of their contractile function can cause a range of diseases such as high blood pressure, asthma, coronary and cerebral artery spasms. In spite of their important role in most bodily functions, the precise mechanism regulating smooth muscle cell contraction is still not completely known. The understanding of the fundamental mechanism of regulation of smooth muscle function is essential for the improvement of treatment of diseases due to abnormalities of smooth muscle contraction.

1.1 Characteristics of the smooth muscle cell

Smooth muscle cells are mononucleate, fusiform and, unlike striated muscle, do not show evident cross-striations by electron or light microscopy. They contain thick, thin and intermediate filaments, but these are not highly organized into sarcomeres as in skeletal and cardiac muscle. The thick and thin filaments, composed mainly of myosin and actin respectively, form the contractile apparatus, whereas the intermediate filament has a structural function (Gabella 1984; Gunst 1999).

The thick filaments are nearly aligned with the long axis of the cell, and surrounded by roughly 15 thin filaments, as observed in vascular muscle. The thin filaments run parallel with the thick filaments and insert acutely into dense areas of plasma membrane. The membrane-associated dense plaques are connected to each other in adjacent cells, constituting the so called attachment plaques, thus conferring mechanical coupling between the cells (Gabella 1984; Gunst 1999). Similar dense bodies are also observed scattered in cytosol of smooth muscle cells. The thin filaments can be seen attached to the opposite ends of these dense bodies, which are believed to be analogous to the Z-line bands of striated muscle, providing anchorage for the thin filaments (Gabella 1984).

The cellular content of actin and tropomyosin in smooth muscle is about twice that of skeletal muscle, but the myosin content in smooth muscle is only one third of that of skeletal muscle. Despite the lower myosin content, smooth muscle is capable of generating the same isometric force per cross-sectional area as the skeletal muscle. Furthermore, smooth muscle contracts slower and maintains tension at a much lower energy cost than skeletal muscle (Eddinger 1998).

The structure of the thick filaments in smooth muscle is supposed to be very different to that in skeletal muscle. Unlike the bipolar arrangement of the thick filaments in skeletal muscle, the smooth muscle thick filaments form a side-polar structure, with the myosin heads facing in the same direction on each side of the molecule, without a central bare zone (Xu 1996).

1.2 Thick filament

1.2.1 Molecular structure of myosin

The thick filaments of smooth muscle are mainly composed of myosin II, the major molecular motor protein of muscle and non-muscle cells, responsible for muscle contraction and cell motility, generated by cyclic interaction with actin. Myosin II is a hexameric molecule, consisting of two heavy chains (MHC), each with a molecular weight of approximately 200 kDa, and two pairs of light chains (MLC). Each MHC has an asymmetric globular amino terminal head domain, linked to a long α -helical tail and a short non-helical C-terminal tailpiece of approximately 34-44 amino acids (Adelstein and Sellers 1996; Gunst 1999).

The myosin heads are composed of a globular motor domain and an α -helical neck region. The motor domain contains the ATP and actin binding domains (Rayment et al. 1996). The function of the neck region of myosin is not completely clear. Besides its association with the myosin light chains, it may act as a rigid “lever arm” to amplify small, nucleotide-mediated conformational changes in the head (Whittaker et al. 1995).

Additionally, a correlation between the length of myosin neck and filament sliding velocity in an *in vitro* motility assay was suggested (Uyeda et al. 1996).

Each myosin head is associated with one essential and one regulatory light chain. Both light chains diverge in their molecular weight, structure and function. Phosphorylation of the 20 kDa MLC (MLC₂₀), also called regulatory or phosphorylatable light chain, triggers the myosin ATPase activity and initiates smooth muscle contraction (Bárány and Bárány 1996; Kamm and Stull 1985). The function of the 17 kDa MLC (MLC₁₇), named essential light chain, is still not clear. It was suggested that it participates in the active site of myosin (Okamoto 1986). In addition, a correlation between the expression of the acidic MLC₁₇ isoform and shortening velocity has been observed (Morano et al. 1993, 1997).

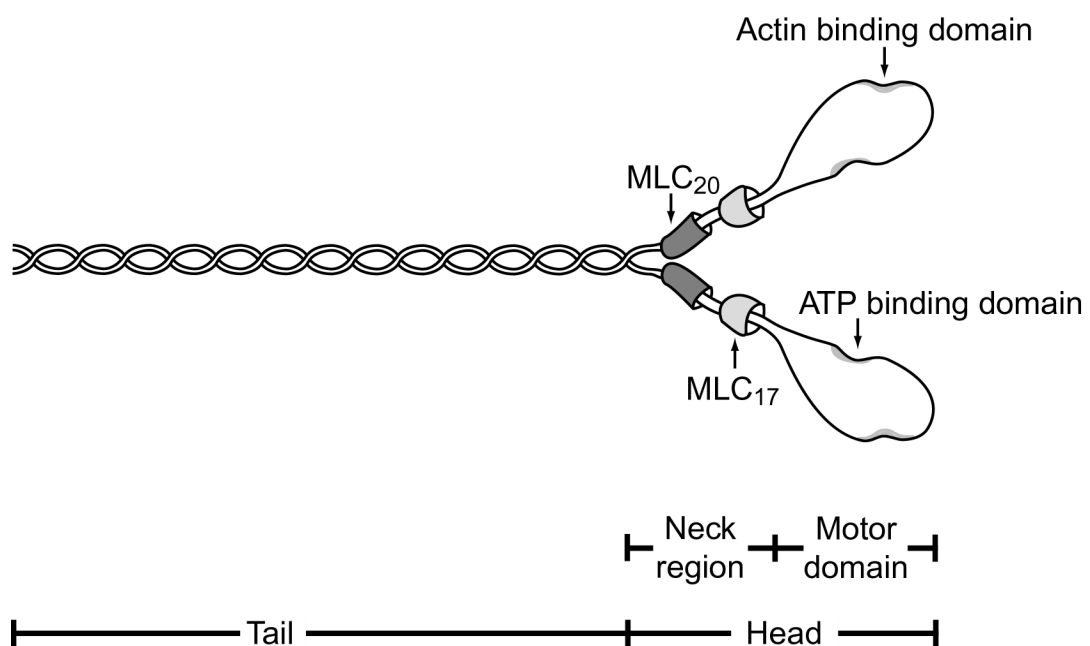


Figure 1. Model of myosin molecule structure, showing the two globular heads, connected to a long α -helical tail. Each head consists of a motor domain, which contains the actin and ATP binding domains, and a neck region, that is associated with MLC₂₀ and MLC₁₇.

The myosin tails are responsible for assembly into functional thick filaments (Trybus 1996). The importance of the tail domain for cellular function of myosin has been investigated with mutant cells that express a truncated form of myosin, similar to the proteolytic fragment heavy meromyosin (HMM). HMM contains the two globular heads and approximately 1/3 of the tail domain. These mutant cells were motile, but showed a great impairment of their general motility and their developmental ability (De Lozanne and Spudich 1987; Wessels et al. 1988), indicating that the assembly portion of the tail domain is essential for the accurate function of myosin. The function of the non-helical tailpiece remains uncertain. It has been suggested that it modulates filament formation in non-muscle myosin (Hodge et al. 1992). However, recent studies have shown that the critical region for filament formation of smooth and non-muscle myosin is the C-terminal end of the coiled-coil tail domain, but not the non-helical tailpiece (Ikebe et al. 2001).

1.2.2 Myosin heavy chain isoforms

Three different genes coding for myosin heavy chain are expressed in smooth muscle cells, namely one for smooth muscle specific myosin heavy chain (SM-MHC) and two genes coding for non-muscle myosin heavy chain (NM-MHC), termed NMA with 196 kDa and NMB with 198/200kDa (Kawamoto and Adelstein 1991; Kelley 1997).

Four mRNA variants of SM-MHC are generated by alternative splicing at the 5' and 3' end of the primary transcript. Alternative splicing of a 39 nucleotide (nt) exon at the 3' end produces two SM-MHC isoenzymes with 204 kDa (SM1) and 200 kDa (SM2) (Nagai et al. 1989; Babij and Periasamy 1989). This exon contains a stop codon. As a result, its inclusion causes the short carboxyl terminus of 9 amino acids of SM2, while splicing of this exon results in the longer carboxyl terminus of 43 amino acids of SM1. Additional alternative splicing of a 21 nt exon at the 5' end close to the ATP binding site generates the inserted seven amino acids (SM1B, SM2B) or non-inserted (SM1A and SM2A) isoforms (Babij et al. 1991; White et al. 1993).

The NM-MHC isoforms A and B are encoded by two separate genes, located on chromosomes 22 and 17 respectively (Saez et al. 1990; Simons et al. 1991). They have

been named non-muscle myosin, since they are not specific for smooth muscle cells, but are also expressed in other non-muscular cell types (Kelley 1997). Two additional NMB isoforms have been reported, generated by alternative splicing in the head region near the actin-binding site (NB1 with 63 nt insertion) and near the ATP-binding site (NB2 with 30 nt insertion). However, these spliced variants are expressed only in nervous tissue, and not in smooth muscle or other non-muscle cells (Takahashi et al. 1992).

Gene sequences of smooth and non-muscle MHC have a high degree of homology. Indeed, smooth and non-muscle myosin are more closely related to each other than to striated myosin (Miano et al. 1994). Both smooth and non-muscle myosin are regulated by phosphorylation and dephosphorylation on conserved sites of the regulatory MLC (mainly at Ser19) by different protein kinases (myosin light chain kinase, Rho-kinase and p21-activated kinase), inducing high motor activity and filament assembly (Bresnick 1999; Sellers et al. 1981; Trybus 1996).

1.2.3 Regulation of myosin isoform expression

The expression of SM-MHC and NM-MHC isoforms varies among smooth muscle tissues, developmental stages and during patho-physiological conditions, suggesting that these isoforms might have different functions. SM-MHC isoforms are exclusively expressed in smooth muscle tissues (Miano et al. 1994), whereas NM-MHC isoforms are variously expressed in different tissues. The expression of myosin isoforms has been studied in rabbits, rats, and humans, and the relative amount of the different isoforms in a given tissue appears to be species-dependent (Aikawa et al. 1993; Frid et al. 1993; Kuro-o et al. 1989 and 1991; Zanellato et al. 1990).

In embryonic and perinatal vascular smooth muscle of rabbit and human, SM1 and NM-MHC are the predominant isoforms expressed, whereas SM2 appears only after birth in rabbit (Kuro-o et al. 1989, 1991), or during late fetal development in humans (Aikawa et al. 1993; Frid et al. 1993). The expression of NM-MHC decreases during postnatal vascular development in parallel with the increase of SM2 expression. NM-MHC markedly increases in proliferating smooth muscle cells of arteriosclerotic plaque (Kuro-o et al.

1991; Zanellato et al. 1990). Additionally, the expression of NM-MHC has been suggested to be associated with the restenosis after coronary atherectomy in humans (Simons et al. 1993).

In rabbit urinary bladder, SM1 is the predominant isoform in newborn. With development, SM1 is down-regulated while SM2 expression increases (Arafat et al. 2001; Chiavegato et al. 1993). NMA isoform is expressed in both adult and newborn bladders, whereas NMB expression is verified at low levels only in newborn bladders (Arafat et al. 2001). In hypertrophied bladder muscle, SM2 is down-regulated in parallel with increase of NMA, but it returns to normal levels with reversal of the hypertrophy (Chiavegato et al. 1993).

In human myometrium, similar amounts of SM1, SM2 and NMA are expressed in the adult state. In the hypertrophied human myometrium, however, NMA decrease is linked to an increase of SM2 (Morano et al. 1997). In rat hypertrophied myometrium, NM-MHC is also down-regulated, but in favor of SM1 (Morano et al. 1993).

The expression of SMA and SMB is also diversely regulated in a tissue-specific manner. In adult rat, SMB is predominantly expressed in small arteries, bladder, intestine, and stomach, whereas SMA is mainly found in aortic and vena cava vascular smooth muscle. In most rat fetal smooth muscle, both SMA and SMB isoforms are expressed, but the expression of SMA is significantly smaller than of SMB (Wetzel et al. 1998; White et al. 1993, 1998).

1.2.4 Functional role of different myosin isoforms

Several studies have reported a functional correlation with different expression of these isoforms in smooth muscle tissues, but a clear relationship remains to be determined. Because of the proximity of the ATP binding site, the 5' insert is believed to have a functional significance. In fact, studies using *in vitro* motility assay revealed that SM-MHC with the 5'-insert (SMB) has a higher Mg²⁺-ATPase activity and moves actin filaments faster than the SM-MHC without 5'-insert (SMA) (Kelley et al. 1993; Rovner et al. 1997). It has been suggested that the extra insertion adjacent to the nucleotide binding site

increases both the rate of ATP binding to and ADP release from cross-bridges (Lauzon et al. 1998), leading to an increase in the shortening velocity. On the contrary, some studies have not found an association between 5' splicing of MHC and speed of smooth muscle shortening (Haase and Morano 1996; Morano et al. 1993, 1997).

In regard to C-terminal SM-MHC isoforms, an increased shortening velocity of myometrium from oestrogen-treated rats has been attributed to an increased amount of SM1 (Hewett et al. 1993). However, Morano et al. (1993) credited the increased shortening velocity of pregnant rat uterus to changes in the expression of MLC isoforms and not to SM1 and SM2 expression. Consistent with this idea, no differences have been found in moving actin filaments by SM1 and SM2 in the *in vitro* motility assay (Kelley et al. 1992). Moreover, experiments with single smooth muscle cells have shown that the different expression of SM1 and SM2 mainly affects the final length to which the cell can contract and not the shortening velocity (Meer and Eddinger 1997).

The interpretation of these studies is further complicated by the diverse association of different head domain isoforms with different tail domain isoforms, as well as with different light chain isoforms, which have also been reported to contribute to differences in myosin kinetic (Murphy et al. 1997). An inverse correlation between the expression of basic MLC₁₇ isoform (MLC_{17b}) and shortening velocity has been noted (Malmqvist and Arner 1991; Matthew et al. 1998). Similarly, an increased relative content of acidic MLC₁₇ isoform (MLC_{17a}) has been observed in parallel with increased shortening velocity (Morano et al. 1993, 1997) and ATPase activity (Helper et al. 1988). However, this correlation in the *in vitro* motility assays is still controversial (Hasegawa and Morita 1992; Kelley et al. 1993; Rovner et al. 1997). In addition, experiments with unloaded shortening velocity in single smooth muscle cells showed no correlation between shortening velocity and MLC_{17a} and MLC_{17b} isoforms expression (Eddinger et al. 2000).

Non-muscle myosin II is known to be directly involved in cytoskeletal rearrangements of non-muscle cells such as cell motility, cell division, cell aggregation and cell morphology (Bresnick 1999; Tan et al. 1992). In smooth muscle cells, however, the functional significance of non-muscle myosin is still unknown. It has been observed that antisense oligonucleotides to NM-MHC suppress smooth muscle cell proliferation *in vitro* (Simons and Rosenberg 1992), suggesting an involvement of non-muscle myosin in the regulation

of smooth muscle cell proliferation. Similar to SM-MHC isoforms, NMA and NMB also differ in their mechanical properties. In the *in vitro* motility assay, it has been observed that NMA has greater Mg^{2+} -ATPase activity and moves actin filaments faster than NMB (Kelley et al. 1996). The true role of non-muscle myosin in smooth muscle cell function, however, remained unknown.

1.3 Thin filament

Monomeric globular actin (G-actin) aggregates to form the thin filaments (F-actin), responsible for interaction with myosin to produce force. F-actin consists of a double α -helical structure of uniformly oriented G-actin. Each G-actin is composed of a single polypeptide with 375 amino acids and a molecular weight of 42 kDa, associated with one molecule of noncovalently bound ATP. The smooth muscle tissues express four different actin isoforms: α and β smooth muscle actin, and γ and δ non-muscle actin. Sequence analysis shows that actin is highly conserved, with only 5% amino acids differing between the diverse isoforms (Gunst 1999; Pollard and Cooper 1986). The expression of different actin isoforms appears to modify during development and patho-physiological conditions (Cavaillé et al. 1986; Eddinger and Murphy 1991; Malmqvist et al. 1991). However, whether the different expression of actin isoforms has a physiological significance concerning the activation of myosin ATPase is still not known.

In addition to actin, the thin filaments contain tropomyosin, caldesmon and calponin, but unlike skeletal and cardiac muscles, do not have troponin.

Tropomyosin has a dimeric α -helical coil structure, arranged along the actin filaments. It binds to F-actin at a ratio of 1 molecule of tropomyosin per 7 monomeric units of actin. The length and the periodicity of tropomyosin in smooth and striated muscle are identical, but its function in smooth muscle is still unknown (Gunst 1999).

Caldesmon (CaD) is a long flexible protein, consisting of a single polypeptide chain with a molecular weight of 93 kDa (Graceffa et al. 1988; Stafford et al. 1994). It has a central rigid domain with an α -helical structure, and a flexible domain at both ends with the actin

binding sites (Mabuchi et al. 1993; Wang et al. 1991a, 1991b). CaD has been shown to bind to F-actin, myosin, tropomyosin and calmodulin (Horowitz et al. 1996; Wang et al. 1991a). It has been observed that CaD can inhibit actin-activated ATPase activity of smooth muscle myosin and actin motility in the *in vitro* motility assay (Hemric and Chalovich 1988; Shirinsky et al. 1992). Its inhibitory activity increases in the presence of tropomyosin and it is also modulated by Ca²⁺-calmodulin, that greatly reduces the affinity of CaD for actin (Shirinsky et al. 1992; Smith et al. 1987). Because of these properties, CaD has been proposed to act as a thin filament regulatory protein, having a similar function to troponin in skeletal muscle (Smith et al. 1987). Additionally, the observation that CaD also binds to myosin has led to the suggestion that CaD acts as a cross-linker of actin and myosin, promoting stabilization of dephosphorylated myosin into thick filaments and thin-thick filament "tethering" (Ikebe and Reardon 1988; Katayama et al. 1995). However, this property has been observed only *in vitro*, and it has not been supported by findings of electron microscopy (Moody et al. 1990).

Calponin (CaP) is a flexible and long protein composed of a single polypeptide chain with a molecular mass of around 30 kDa (Stafford et al. 1995; Takahashi et al. 1986). It binds to F-actin and myosin (Horowitz et al. 1996; Winder and Walsh 1993). CaP has been shown to inhibit actomyosin ATPase activity and actin filament motility *in vitro*, but in contrast to caldesmon, tropomyosin does not increase the inhibitory activity of CaP (Shirinsky et al. 1992; Winder and Walsh 1990). It is localized in both cytoskeletal and contractile domains (North et al. 1994b), but studies with immunoelectron microscopy have shown a preferential localization of CaP at the periphery of cytoskeletal domain in association with desmin (Mabuchi et al. 1996).

1.4 Smooth muscle contraction

Smooth muscle contraction is thought to occur primarily according to the cross-bridge cycling model presented for striated muscle (Huxley 1957): it was suggested that smooth muscle contracts through sliding of adjacent thick and thin filaments against to each other, without changes in the length of the filaments themselves, driven by the cyclic interaction of myosin with actin (Kamm and Stull 1985).

1.4.1 Actin-myosin interaction

The smooth muscle contraction results from cyclic interaction of myosin cross-bridges with actin, energized by ATP hydrolysis at the globular myosin head (Arner and Pfitzer 1999).

In the absence of ATP, the head portion of the myosin (M) molecules attaches with high affinity to actin (A) filaments. The binding of ATP to myosin (step 1) reduces its affinity for actin, that leads to detachment of myosin from actin (step 2). Free myosin hydrolyzes ATP to ADP and P_i (step 3) and myosin-products complex reattaches to actin in a weak binding state (step 4). As actin rebinds to myosin, P_i is released (step 5), leading to a conformational change, which makes the myosin pull against the actin filament, followed by isomerisation (step 6) and ADP release (step 7). Thus, the transduction of chemical energy into directed movement occurs during product release and not during the hydrolysis step (Arner and Pfitzer 1999; Rayment et al. 1996).

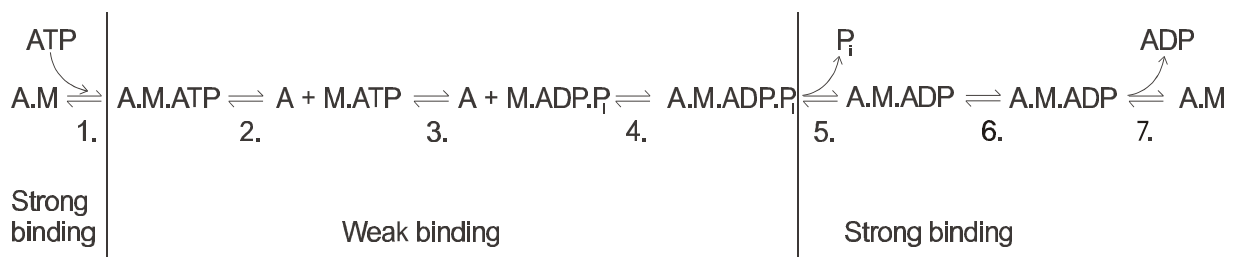


Figure 2. Cross-bridge cycle

1.4.2 Activation of smooth muscle contraction

A rise in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is the main mechanism for initiating contraction in both striated and smooth muscle cells (Filo et al. 1965). However, the mechanisms whereby Ca^{2+} activates contraction vary essentially between the different muscle types. In smooth muscle cells, the increased $[\text{Ca}^{2+}]_i$ binds to calmodulin (CaM) and the Ca^{2+} -calmodulin complex activates myosin light chain kinase (MLCK). Activated myosin light chain kinase phosphorylates the regulatory light chains of myosin (MLC_{20}) mainly at Ser-19 (Gallagher et al. 1997; Ikebe and Hartshorne 1985). Phosphorylation of MLC_{20} leads to myosin-actin interaction, increase in myosin ATPase activity, and smooth muscle contraction (Kamm and Stull 1985).

The decrease in $[\text{Ca}^{2+}]_i$ results in dissociation of Ca^{2+} .CaM.MLCK complex, leading to inactivation of MLCK, thus permitting dephosphorylation of MLC_{20} by myosin light chain phosphatase (MLCP), deactivation of actomyosin ATPase and initiation of smooth muscle relaxation (Kamm and Stull 1985).

1.4.3 Regulation of intracellular Ca^{2+} concentration

The increase of $[\text{Ca}^{2+}]_i$ during contraction results from influx of Ca^{2+} through voltage-dependent channels and from Ca^{2+} -released from intracellular stores (Bolton et al. 1999).

In smooth muscle cells, two types of voltage-dependent Ca^{2+} -channels have been described, T-type (transient) and L-type (long lasting) channels (Bolton et al. 1999). Depolarization of cell membrane by action potentials or increase in extracellular K^+ is the main mechanism of L-type Ca^{2+} -channel activation, promoting its change from the closed to the open state. The smooth muscle cells can also be depolarized by agonists directly through the opening of nonspecific cation channels and inhibition of K^+ channels, or indirectly through activation of Cl^- channels by increased intracellular Ca^{2+} , which results from agonist-dependent Ca^{2+} release from intracellular stores (Rembold 1996).

The release of Ca^{2+} from intracellular stores is mediated by the action of inositol 1,4,5-

trisphosphate (InsP_3) and by Ca^{2+} -induced Ca^{2+} release. InsP_3 is produced through activation of phospholipase C (PLC) by the binding of agonists to receptors coupled to G-proteins. Activated PLC hydrolyzes a membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), to InsP_3 and 1,2-diacylglycerol (DAG). InsP_3 translocates from the plasma membrane to a specific receptor on the sarcoplasmic reticulum, thereby triggering Ca^{2+} release from the intracellular store (Bolton et al. 1999; Rembold 1996). The physiological role of InsP_3 in Ca^{2+} release in smooth muscle cells has been extensively confirmed through the use of heparin, an inhibitor of InsP_3 -receptor (Kobayashi et al. 1989); through stimulation of skinned smooth muscle with InsP_3 , which leads to Ca^{2+} release and subsequent contraction (Somlyo et al. 1985); and through kinetic studies showing InsP_3 release followed by increase in Ca^{2+} and contraction (Somlyo et al. 1992). The Ca^{2+} release induced by InsP_3 is also modulated by Ca^{2+}_i itself, that potentiates the opening of InsP_3 -receptor channel if $[\text{Ca}^{2+}]_i$ is below 300 nM, but inhibits it above this concentration (Iino and Tsukioka 1994).

It has been also suggested that Ca^{2+} -induced Ca^{2+} release through activation of ryanodine receptors participates in Ca^{2+} -regulation of smooth muscle cells (Herrmann-Krank 1991; Xu et al. 1994). However, its existence under physiological conditions remains unclear. In contrast, the influence of $[\text{Ca}^{2+}]_i$ in InsP_3 -mediated Ca^{2+} release mentioned above might functionally act as a Ca^{2+} -induced Ca^{2+} release-like mechanism in a specific range of $[\text{Ca}^{2+}]_i$.

1.4.4 Regulation of smooth muscle contraction

A rise in $[\text{Ca}^{2+}]_i$ and a subsequent Ca^{2+} .CaM.MLCK-dependent phosphorylation of MLC_{20} is the main triggering mechanism for activation of smooth muscle contraction (Somlyo and Somlyo 1994). However, it has been observed that after the initial increase in $[\text{Ca}^{2+}]_i$, the levels of $[\text{Ca}^{2+}]_i$ and MLCK activity decline to near-resting levels, but MLC_{20} phosphorylation and force are sustained. Furthermore, stimulation of smooth muscle by several agonists induces MLC_{20} phosphorylation and contraction without a proportional increase in $[\text{Ca}^{2+}]_i$, the so called Ca^{2+} -sensitization. Thus, it has been suggested that

modulation of the sensitivity of MLC_{20} phosphorylation to Ca^{2+} is an additional meaningful mechanism of regulation of smooth muscle contraction (Somlyo and Somlyo 1994). It is generally believed that the main mechanism of Ca^{2+} -sensitization is through inhibition of MLCP, with alteration of the balance MLCK/MLCP in favor of MLCK, which would lead to increase MLC_{20} phosphorylation at a given $[\text{Ca}^{2+}]_i$ (Kitazawa et al. 1991; Somlyo and Somlyo 1994).

Smooth muscle MLCP is composed of three subunits: a 110-130 kDa regulatory subunit ($M_{110-130}$), a 37-38 kDa catalytic subunit (PP-1C), and a 20 kDa subunit of yet undetermined function (Hartshorne et al. 1998; Mitsui et al. 1992; Shirazi et al. 1994). The $M_{110-130}$ subunit is responsible for selectivity for myosin, binding to both phosphorylated myosin and PP-1C, and additionally for regulation of activity of PP-1C (Hartshorne et al. 1998; Shirazi et al. 1994).

The main pathways proposed for MLCP inhibition involve protein kinase C, RhoA/Rho-kinase and arachidonic acid.

1.4.4.1 Protein kinase C

The involvement of protein kinase C (PKC) in smooth muscle contraction was firstly suggested by the observation that tumor-promoting phorbol esters, which specifically activate PKC, induce slow sustained contractions in several types of smooth muscle (Chatterjee and Tejada 1986; Danthuluri and Deth 1984). Additionally, many agonists have been shown to induce sustained increases in 1,2-diacylglycerol (DAG), the physiological activator of PKC (Griendling et al. 1986; Takuwa et al. 1986). Furthermore, recent studies using specific PKC inhibitors in intact and permeabilized smooth muscle have confirmed the role of PKC in sustained contraction under physiological conditions (Lee et al. 1999; Murthy et al. 2000).

DAG is generated through phospholipase C (PLC)-mediated phosphatidylinositol 4,5-biphosphate (PIP_2) hydrolysis and by phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PC). Unlike PIP_2 hydrolysis, hydrolysis of PC does not result in the

release of inositol 1,4,5- trisphosphate (InsP₃) and consequently there is no release of Ca²⁺ from intracellular stores (Lee and Severson 1994). Some studies have shown that PC hydrolysis is the major source for sustained DAG formation in agonist-stimulated smooth muscle cells (Lee and Severson 1994). During tone maintenance, low [Ca²⁺]_i levels are observed. Thus, the stimulation of PKC, mediated by PC hydrolysis, is consistent with the sustained phase of agonist-induced contraction.

It has been shown that PKC can phosphorylate MLC₂₀ at Ser-1, Ser-2 and Thr-9 *in vitro* (Ikebe et al. 1987). However, the PKC-induced phosphorylation of MLC₂₀ is not correlated with increase of myosin ATPase activity, or with filament movement in the *in vitro* motility assay (Ikebe et al. 1987; Umemoto et al. 1989). In addition, stimulation of smooth muscle with agonists known to activate PKC is not accompanied by phosphorylation of MLC₂₀ at PKC-specific sites (Singer et al. 1989). In contrast, the mechanism whereby PKC mediates Ca²⁺-sensitization may be through inhibition of myosin light chain phosphatase (Itoh et al. 1993; Masuo et al. 1994). Indeed, recent studies have shown that PKC phosphorylates CPI-17, an endogenous inhibitor of MLCP. PKC-induced phosphorylation of CPI-17 has been shown to induce Ca²⁺-sensitization through inhibition of MLC₂₀ dephosphorylation, supporting CPI-17 as a physiological mediator of PKC-induced Ca²⁺-sensitization in smooth muscle (Eto et al. 1995; Kitazawa et al. 2000; Li et al. 1998; Senba et al. 1999).

Additionally, atypical PKC isoforms activated by arachidonic acid have also been shown to inhibit MLCP activity (Gailly et al. 1997). Furthermore, PKC is also believed to participate in thin filament regulatory mechanism of smooth muscle contraction. PKC may directly or indirectly phosphorylate the thin filament associated proteins caldesmon and calponin, which would relieve their inhibitory function, allowing actin-myosin interaction and contraction (Horowitz et al. 1996; Rokolya et al. 1994; Winder and Walsh 1993).

1.4.4.2 RhoA/Rho-kinase pathway

The RhoA/Rho-kinase pathway is also believed to play a significant physiological role in Ca²⁺-sensitization. Experiments with permeabilized smooth muscle have shown a Ca²⁺-

sensitization effect of GTP-bound RhoA, which was blocked by ADP-ribosylation of RhoA (Hirata et al. 1992). Moreover, the highly specific antagonist of Rho-kinase, Y-27632, inhibited the tonic phase of agonist-induced contractions in intact and permeabilized smooth muscle (Fu et al. 1998; Uehata et al. 1997).

RhoA is a member of the Rho family of low molecular weight monomeric G-proteins. It is active in GTP-bound and inactive in GDP-bound forms (Nobes and Hall 1994). In resting smooth muscle, most of RhoA is localized in cytosol, associated with RhoGDI (guanine nucleotide dissociation inhibitor). Following the stimulation of G-protein coupled receptors by some agonists, RhoA.RhoGDI is activated by Rho-GEFs (guanine nucleotide exchange factors) that catalyze GTP-GDP exchange on RhoA, succeeded by translocation of RhoA.GTP to the cell membrane (Gong et al. 1997). In plasma membrane, RhoA.GTP activates Rho-kinase, which subsequently phosphorylates the regulatory subunit of MLCP, $M_{110-130}$, resulting in inhibition of the MLCP activity (Feng et al. 1999b; Kimura et al. 1996; Nobes and Hall 1994). Furthermore, recent studies have shown that Rho-kinase can also phosphorylate and activate CPI-17, an endogenous inhibitor of MLCP (Koyama et al. 2000).

1.4.4.3 Arachidonic acid

Arachidonic acid, released by certain agonists, is also considered as a Ca^{2+} -sensitizing agent. It has been shown to inhibit MLCP directly *in vitro* through dissociation of the regulatory from the catalytic subunit of MLCP, leading to increased MLC_{20} phosphorylation at constant Ca^{2+} (Gong et al. 1992). More recent studies have shown that arachidonic acid can also activate Rho-kinase, independently of RhoA (Araki et al. 2001; Feng et al. 1999a; Fu et al. 1998). Moreover, arachidonic acid has been shown to activate atypical PKC isozymes, thereby inhibiting MLCP activity (Gailly et al. 1997).

1.4.4.4 Thin filament regulation

Thin filament regulation is another mechanism proposed to explain the maintenance of tone despite low MLC₂₀ phosphorylation levels observed in smooth muscle. It is believed that the proteins responsible for thin filament regulation are caldesmon and calponin, since both molecules have been shown to bind actin and inhibit actomyosin ATPase *in vitro* (Hemric and Chalovich 1988; Shirinsky et al. 1992; Winder and Walsh 1990).

With the observation that caldesmon (CaD) can inhibit myosin ATPase and cross-link actin and myosin *in vitro*, a dual function for CaD has been proposed (Horowitz et al. 1996). According to this model, CaD is attached to actin, leading to a decrease of the affinity between actin and myosin. Binding of CaD to myosin promotes stabilization of the dephosphorylated thick filaments. Upon stimulation, CaD then detaches from the MLC₂₀-phosphorylated thick filaments and switches the thin filament into the “on-state”, thus permitting muscle contraction. During tone maintenance, CaD reappears to “tether” the thick and thin filaments together and to stabilize the dephosphorylated myosin filaments. The inhibitory function of CaD could be relieved by CaD phosphorylation, induced by PKC, mitogen-activated protein kinase or by binding to Ca²⁺-calmodulin (Adam and Hathaway 1993; Smith et al. 1987; Tanaka et al. 1990). Although many *in vitro* studies suggest the regulatory role of CaD in smooth muscle contraction, their *in vivo* function is still not confirmed (Horowitz et al. 1996).

Calponin has also been observed to bind to actin and to inhibit actin-activated myosin ATPase and actin filament movement over phosphorylated myosin in the *in vitro* motility assay (Shirinsky et al. 1992; Winder and Walsh 1990). It has been proposed that its inhibitory activity would be relieved by phosphorylation, induced by PKC, Ca²⁺-calmodulin-dependent kinase II or other Ca²⁺-binding proteins (Winder and Walsh 1990, 1993). However, its physiological role in smooth muscle contraction is still unclear, since most data has been achieved from *in vitro* studies. In addition, its predominant distribution in the cytoskeletal domain rather than in the contractile apparatus suggests that it does not function as a contractile regulatory protein, but that it possibly promotes the interaction between actin and cytoskeletal proteins (Mabuchi et al. 1996). Moreover, a recent study using a calponin knockout mouse model showed no difference in the Ca²⁺- and agonist-

induced isometric force in the smooth muscle preparations in the absence of calponin (Matthew et al. 2000).

1.4.4.5 Latch-bridges hypothesis

The “latch” concept originated from the observation of force maintenance in arterial smooth muscle, despite low levels of phosphorylation in parallel with decreased shortening velocity (Dillon et al. 1981). According to this hypothesis, dephosphorylation of already attached cross-bridges would decrease the detachment rate, forming a “latch bridge”. Thus, latch-bridges are attached, dephosphorylated, slowly cycling cross-bridges, that sustain force but inhibit shortening (Dillon et al. 1981). Besides dephosphorylation of previously attached cross-bridges, a “cooperative attachment” of dephosphorylated cross-bridges may also contribute to the latch phenomenon. Thus, the attachment of cross-bridges would increase affinity and attachment of the neighboring cross-bridges to actin (Somlyo et al. 1988).

This concept is consistent with observations that phosphorylation of MLC_{20} increases the release of P_i from actin-myosin-ADP- P_i forming the strongly attached actin-myosin-ADP state (Greene and Sellers 1987), and that tonic smooth muscle myosin has a high affinity for ADP (Fuglsang et al. 1993). However, the latch bridge could not be isolated biochemically until now, and the possibility of a positive-feedback situation would lead to an ascending contraction, which argues against the existence of this model (Murphy 1994).

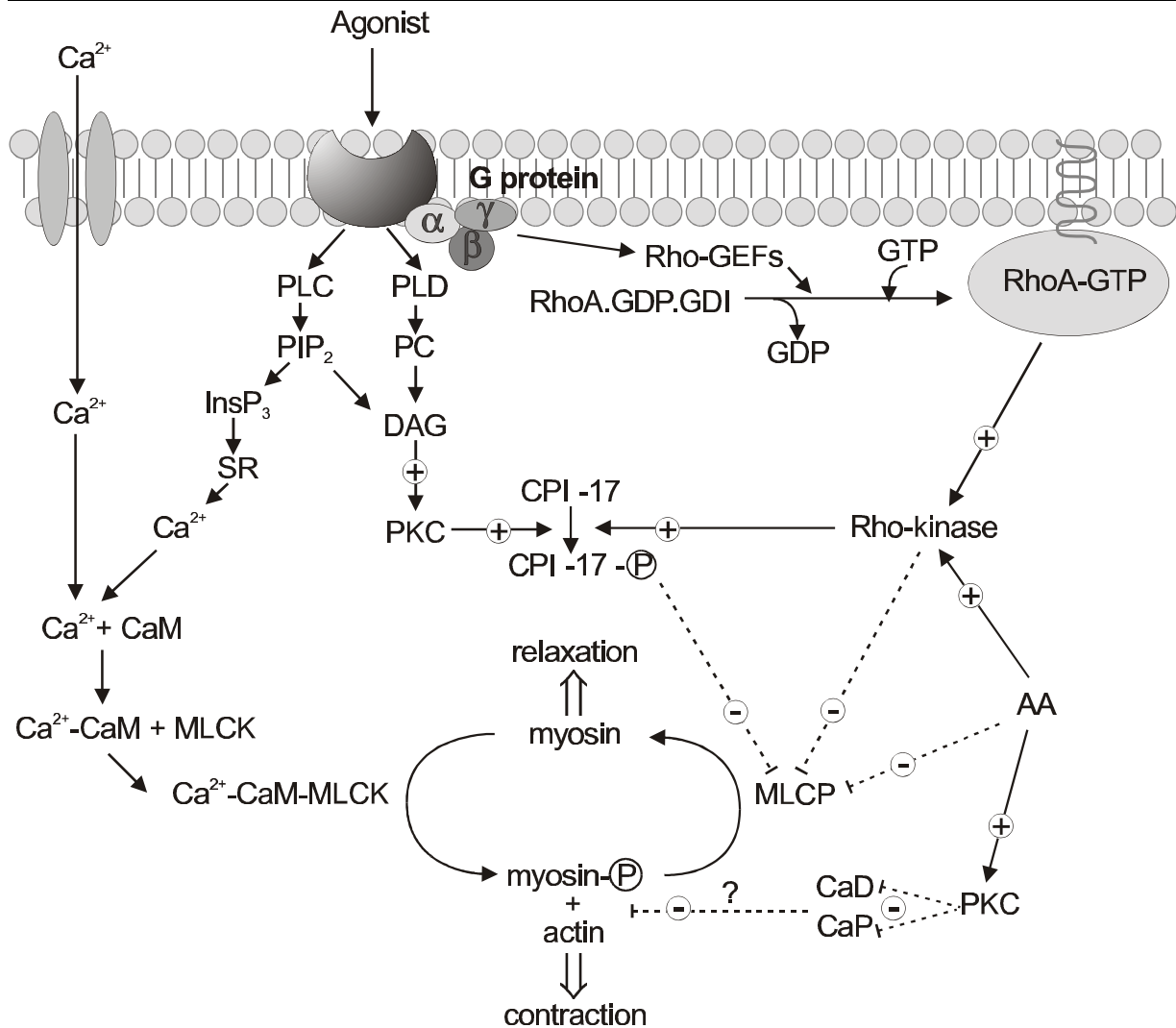


Figure 3. Proposed models for the regulation of smooth muscle contraction. The increased intracellular Ca^{2+} concentration binds to calmodulin (CaM) and the calcium-calmodulin complex activates myosin light-chain kinase (MLCK) to phosphorylate the regulatory light chains of myosin, leading to myosin-actin interaction and contraction. Phospholipase C- (PLC) and D- (PLD) mediated membrane phospholipids hydrolysis leads to activation of protein kinase C (PKC), resulting in phosphorylation of CPI-17 and myosin light phosphatase (MLCP) inhibition. Activation of G-protein coupled receptors also activates Rho-kinase, which can directly or indirectly inhibit MLCP activity. Arachidonic acid (AA) can also activate Rho-kinase and PKC and also appears to directly inhibit MLCP. Abbreviations: CaD, caldesmon; CaP, calponin; DAG, diacylglycerol; InsP₃, inositol 1,4,5-trisphosphate; SR, sarcoplasmic reticulum; PIP₂, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine.

1.5 Aim of the study

Despite the knowledge of many smooth and non-muscle myosin isoforms present in smooth muscle tissues, it is not clear to what extent the different myosin heavy chain isoforms contribute to their contractile features. Especially, there is no evidence of the participation of non-muscle myosin in smooth muscle contraction until now. In order to determine the physiological significance of smooth muscle specific and non-muscle myosin heavy chain in smooth muscle contraction, smooth muscle preparations from a mouse model with a disrupted smooth muscle myosin heavy chain gene were used.

The expression of smooth muscle and non-muscle myosin heavy chain was evaluated by Western blot analysis using specific anti-smooth muscle and anti-non-muscle myosin antibodies. Furthermore, the presence of intracellular filamentous structures was investigated in cultured smooth muscle cells by immunofluorescence using a double labeling method with specific antibody against smooth muscle and non-muscle myosin heavy chain and an antibody against smooth muscle α -actin. Finally, the mechanical analysis was performed by using intact muscle preparations, which provides a good model for functional studies under physiological conditions.

Thus, it was possible - for the first time - to verify the importance of the non-muscle myosin heavy chain isoforms for smooth muscle contraction *in vivo*.

MATERIALS

2.1 Equipment

Amersham Pharmacia Biotech, Amersham Place, England

Nitrocellulose membrane, Hybond ECL

YT Recorder REC 111

Bio-Rad, Munich

Mini-PROTEAN II Slab Cell

Power supply model 1000/500

HAMEG, Frankfurt am Main

Oscilloscope HM 408

Graphic Printer HD 148

KODAK, New York

X-ray film

Leica, Heerbrugg, Switzerland

Stereomicroscope wild M3Z

Schott, Wiesbaden

Cold-light sources KL 750

Scientific Instruments, Heidelberg

MLK1 system

MLK1M system

Shimadzu Europa, Duisburg

Spectrophotometer UV-1202

Whatman, Maidstone, England

3MM Chr. Chromatography paper

Zeiss, Jena

Axioplan fluorescence microscope

MC100 automatic camera

2.2 Chemicals and drugs

Amersham Pharmacia Biotech, Amersham Place, England

Western blotting detection reagents, ECL

Biochrom, Berlin

Fetal calf serum (FCS)

Gentamicin

Penicillin G

Streptomycin

Bio-Rad, Munich

Bovine gamma globulin standard

Prestained SDS-PAGE Standards, high range

Protein assay dye reagent concentrate

Tween 20

Boehringer, Mannheim

Anti- α -actin (clone asm-1)

4',6-Diamidin-2'-Phenylindol Dihydrochlorid (DAPI)

Calbiochem-Novabiochem, Bad Soden

Ro-31-7549

Dianova, Hamburg

Cy3-conjugated anti-mouse IgG

DTAF-conjugated anti-rabbit IgG

Gibco BRL, Paisley, Scotland

DMEM

Merck, Darmstadt

Hydrochloric acid (HCl) fuming 37%

KH_2PO_4

MgSO_4

NaHCO_3

Serva, Heidelberg

Acrylamide 2x

Albumin bovine serum Fraction V (BSA)

CaCl₂

Dimethyl sulfoxide (DMSO)

Dithiothreitol (DTT)

Glucose

Glycine

Glycerol

N,N'-methylene-bis acrylamide

Ponceau S

Sodium dodecylsulfate (SDS)

Tris(hydroxymethyl)aminomethane (Tris)

Sigma, Steinheim

Albumin, chicken egg

Anti-mouse IgG

â-mercaptoethanol

Bromophenol blue

2,3-Butanedione monoxime (BDM)

Collagenase F

Collagenase H

Na-glutamat

NaN_3

Papain

Phorbol 12,13-Dibutyrate

Reanal, Budapest, Hungary

N,N,N,N-Tetramethyl-ethylene diamine (TEMED)

Roth, Karlsruhe

Ammonium-peroxodisulfat (APS)

Methanol

KCl

NaCl

Sodium lauryl sulfate (SDS)

METHODS

3.1 Animal model

As experimental model, urinary bladder preparations from neonatal wild-type C57BL6 mice as well as from neonatal smooth muscle myosin heavy chain (SM-MHC) deficient mice of both sexes were employed. The SM-MHC deficient mice were created through gene-targeting technology, leading to total depletion of SM-MHC isoforms (Morano et al. 2000).

The SM-MHC deficient mice were delivered at the expected Mendelian ratio and had a normal body weight, suggesting normal fetal development. Most of the SM-MHC deficient mice died within 12 and 24 hours of life. For this reason, all experiments were performed with neonatal mice in the first days after birth.

3.2 Biochemical methods

The expression of SM-MHC and non-muscle myosin heavy chain (NM-MHC) isoforms at the protein level in bladders of wild-type and SM-MHC deficient mice was evaluated by Western blot analysis with specific anti-SM-MHC and anti-NM-MHC antibodies.

Urinary bladder was homogenized in lysis buffer, centrifuged at 12000 rpm for 5 min at 4°C. The supernatant was frozen in liquid nitrogen and stored at -80°C until use. A small aliquot was kept for protein determination.

Lysis buffer consists of SDS 5%; Tris-HCl 50 mM, pH 7,5; saccharose 0.25 M; urea 75 mM; 2-βmercaptoethanol 60 mM.

3.2.1 Protein determination

The protein content was determined using the Bradford assay. A standard curve was constructed using α -IgG (Bio-Rad). The unknown sample (5 to 40 μ l) was mixed with 200 μ l of protein reagent (Bio-Rad) and 800 μ l distilled water and allowed to stand for 10 min. The sample was subsequently read in a photometer at 595 nm. Protein concentration of the unknown sample was determined from the standard curve.

3.2.2 Electrophoretic separation of myosin heavy chain isoforms

Sample buffer contains 0.5 M Tris-HCl, pH 6.8 12,5% (v/v); glycerol 10% (v/v); SDS 2% (w/v); 2- β mercaptoethanol 5% (v/v); bromophenol blue 0,001% (w/v).

SDS electrophoresis buffer contains Tris base 0.025 M; glycine 0.192 M; and SDS 0.1% (w/v).

All solutions were prepared with bi-distilled water.

The myosin heavy chain isoforms were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), according to a modified Laemmli gel method (Laemmli 1970). The Mini-Protean II dual slab cell (Biorad) was used, which provides a gel with the following dimensions: 80 mm wide, 70 mm long and 1.5 mm thick. A 5% separating gel (see table 1) was prepared and applied between the glass plates. The solution was covered with distilled water to provide a flat gel surface. After polymerization, a 4% stacking gel (see table 1) was prepared and applied above the separating gel, after the water was poured off. A comb was placed into the stacking gel solution, and it was allowed to polymerize. After polymerization, the comb was removed and each well was loaded with 25 μ g protein. The control wells were loaded with the high-molecular weight standard kit of Bio-Rad. The protein samples were diluted 1:4 (v/v) with sample buffer and heated 5 min at 95°C.

	Separating gel	Stacking gel
Distilled water	5.675 ml	3.05 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	—
0.5 M Tris-HCl, pH 6.8	—	1.25 ml
30% Acrylamide/ 0.8% bis	1.67 ml	0.65 ml
SDS 10%	100 μ l	50 μ l
APS 10%	50 μ l	25 μ l
Temed	5 μ l	5 μ l

Table 1. Recipes for separating and stacking polyacrylamide gels, based on the buffer system of Laemmli.

Electrophoresis was carried out at 220 V. The temperature was kept at 15°C. The gels were processed for Western blot analysis.

3.2.3 Western blot analysis

Transfer buffer consists of 25 mM Tris base; 192 mM glycine and methanol 20% (v/v). Blocking buffer contains 15% ovalbumin; 0.1% Tween; 20 mM Tris base and 137 mM NaCl.

TBS includes 20 mM Tris base, 137 mM NaCl. The pH was adjusted to 7.6 with HCl. TBST consists of TBS added with Tween 0.1%.

All solutions were prepared with bi-distilled water.

Following SDS-PAGE, the gel was equilibrated for 10 minutes at room temperature in transfer buffer. The proteins were electrophoretically transferred from gel to nitrocellulose membrane (Hybond-C, Amersham), for 2 hr at 250 mA. Following transfer, the

membrane was incubated with Ponceau staining solution for 10 minutes at room temperature. The nitrocellulose membrane was blocked with 15% ovalbumin and incubated with a polyclonal antibody raised against chicken-gizzard SM-MHC (1:100,000) or with a polyclonal antibody raised against the rod-portion of NM-MHC (1:1250; both antibodies gift from Prof. Gröschel-Steward, Darmstadt, Germany) overnight at 4°C. Subsequently, the membranes were incubated with the secondary peroxidase-conjugated antibody (1:6000 and 1:6500, respectively) for 1 h at room temperature. Between each step, the membranes were washed by agitating with TBS and TBST solutions. Signals were then visualized by the enhanced chemiluminescence reaction kit (ECL; Amersham) using X-ray film (X-Omat; Kodak).

3.3 Immunofluorescence staining of cultured smooth muscle cells

3.3.1 Cell culture

Hepes buffer used for cell culture included 55 mM NaCl, 5.6 mM KCl, 80 mM Na-glutamat, 2 mM MgCl₂, 10 mM glucose and 10 mM Hepes. NaOH was used to adjust pH to 7.4. Bovine serum albumin (BSA, 1mg/ml), penicillin (0.3 mg/ml) and streptomycin (0.5 mg/ml) were further added in the Hepes buffer.

Papain 0.5 mg and DTT 1 mg were dissolved in 1 ml of Hepes buffer. Collagenase F and H were dissolved in Hepes buffer with the ratio F/H of 70%/30%, to give a final concentration of 1 mg/ml, and 0.01mM CaCl₂ was added.

All solutions and materials used directly in contact with the cells were sterile.

Primary cell culture of urinary bladder was performed by a modification of the procedures described by Gollasch et al. (Gollasch et al. 1992). Neonatal mice were killed by decapitation and the bladders were excised and placed in Hepes buffer, added with BSA, penicillin and streptomycin. Adhering fat and connective tissue were removed by

dissection under microscope and the bladders were minced into small pieces. The resulting bladder pieces were placed in papain/DTT solution and incubated for 25 min at 36°C. The cell-tissue suspension was centrifuged (900 rpm, 4 min) and the following cell pellet incubated with collagenase F and H for 15 min at 36°C. After centrifugation (900 rpm, 4 min), the cell pellet was resuspended in DMEM medium containing 20% of fetal calf serum (FCS) and gentamicin (0.02 mg/ml). Cells were then seeded in plastic culture dishes and cultivated for 24 hours at 37°C in a humidified atmosphere of 5% CO₂/95% air.

3.3.2 Immunofluorescence labeling

Cells were fixed with methanol/acetone (50% v/v) for 10 minutes at -20°C. Double immunolabeling was carried out with an antibody against smooth muscle α -actin (aS-actin; clone asm-1, Boehringer) and a polyclonal antibody raised against the rod-portion of NM-MHC (aNm-MHC). In another set of experiments, double labeling was performed with a polyclonal antibody raised against chicken-gizzard MHC (aSM-MHC) and with aS-actin (see above). Secondary antibodies were conjugated with DTAF (anti-rabbit) or Cy3 (anti-mouse). Nuclei were simultaneously stained by DAPI.

Before each incubation with the antibodies, the cells were pre-incubated with a solution containing 20 mM Tris-HCl, pH 8.4, 630 mM NaCl, 0.05% Tween 20%, 0.02% NaN₃, and 1% BSA-Tris for 30 minutes at room temperature to suppress nonspecific labeling. The same solution was used to dilute both primary and secondary antibodies. Incubation with the primary antibodies was executed for 1 hour at 37°C, and with the secondary antibodies for 30 minutes at 37°C. Control staining was performed with the secondary antibody alone and minimal background has been observed.

Fluorescence was detected using an Axioplan fluorescence microscope with appropriate filter systems. Micrographs were followed with an MC100 automatic camera with Kodak Tmax 400 film.

3.4 Mechanical analysis of smooth muscle preparations

3.4.1 Solutions

Krebs-Henseleit solution used for intact muscle preparation contained 119 mM NaCl, 12.2 mM glucose, 4.6 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.0 mM CaCl₂, 2.0 mM Na-pyruvate, pH 7.4. The solution was gassed with a mixture of 96% O₂ and 4% CO₂ throughout the experiments.

The strips of smooth muscle were prepared in Krebs-Henseleit solution added with 5 mM 2,3-butanedione monoxime (BDM).

The muscle preparations were activated in KCl depolarization solution or with addition of phorbol diester in Krebs-Henseleit solution. The KCl depolarization solution was made with equimolar replacement of NaCl by 50 mM KCl in Krebs-Henseleit solution.

The phorbol diester chosen was phorbol 12, 13-Dibutyrate (PDBu), since this drug has been reported to be effective in intact muscle preparations (Yoshida et al. 1992). PDBu was dissolved in dimethylsulfoxide (DMSO), and then diluted to the required concentrations with distilled water. Final concentrations of DMSO were not greater than 0.1% and did not have significant effects on the contractile response to PDBu.

In some experiments, the muscle preparations were incubated with a specific, cell-permeable protein kinase C inhibitor (Ro-31-7549), before stimulation with PDBu.

3.4.2 Smooth muscle preparations

Intact urinary bladder strips were prepared from neonatal wild-type as well as from neonatal SM-MHC deficient mice. The animals were killed by decapitation and the bladders were quickly taken out and placed in Krebs-Henseleit solution added with BDM. The bladders were excised and longitudinal strips of the posterior wall of the bladder were prepared by using micro scissors under a dissecting microscope. The strips were

approximately 1 mm long and 0.4-0.5 mm wide. During all the procedure the solution was continuously oxygenated.

3.4.3 Isometric force registration

3.4.3.1 Description of mechanical and electronic equipment

The MLK1 system from Scientific Instruments was used for isometric force measurements in intact muscle preparations. In this system, the muscle preparations are mounted horizontally using micro-clamps between a force transducer at one end and a micrometer at the other end in a tissue bath. The tissue bath chamber is connected to another chamber, where the incubation solution is oxygenated. This two-chamber system provides an oxygenation process with minimal interference in the force measurement, since the gas bubbles do not have direct contact with the incubation solution. The incubation solution temperature is controlled by a thermostat coupled to the block containing the solution chambers and is constantly checked with a thermometer. Temperature was 37°C. After the muscle preparations are mounted, the fibre length can be precisely adjusted using a micrometer screw, in order to reach the desired resting tension.

This system is connected to a force transducer and a YT recorder. To calibrate the force transducer, a piece of wire weighting 509.7 mg was hung over the tip of the transducer.

3.4.3.2 Experimental design

The urinary bladder preparations were mounted isometrically in the tissue bath containing 5 ml of Krebs-Henseleit solution and equilibrated for 60 min before the beginning of the experiments. The tissue bath was continuously gassed with 96% O₂ and 4% CO₂ and the temperature was kept at 37°C. Resting tension was adjusted to 0.3 mN.

For isometric force development, bladder preparations were activated in KCl

depolarization solution or stimulated with PDBu, and force was registered using the YT-recorder. The isometric force was obtained as total minus passive force (mN).

For the experiments with the protein kinase C inhibitor, the muscle preparations were first equilibrated in Krebs-Henseleit solution, then incubated with the inhibitor for 20 minutes, before being stimulated with PDBu.

3.4.4 Isotonic contraction

3.4.4.1 Description of mechanical and electronic equipment

For force-velocity determinations, isotonic quick releases under constant load were applied to the preparations during steady state isometric contraction using the system MLK1M from Scientific Instruments. In this system, the muscle preparation is mounted isometrically between a force transducer and a linear motor using micro-clamps, immersed in oxygenated solution in a two chamber system, connected to a thermostat. Temperature was 37°C. The muscle strips were stimulated with 50 mM KCl to generate isometric force.

The constant load unit allows the adjustment of load steps between 3% and 100% of the isometric force. Load clamping for isotonic shortening is achieved by changing the mode of operation of the motor from fibre-length control to force control by a trigger signal.

The length signal was additionally amplified 10 times using an external amplifier, in order to increase the confidence of the length-change measurement.

The system is connected to a PC and the signals were stored and evaluated with MUSKI software (Scientific Instruments) that controls the selected variables.

The system is also connected to a YT recorder, where isometric force and releases were continuously recorded.

3.4.4.2 Experimental design

The muscle preparations were mounted isometrically in 5 ml of Krebs-Henseleit solution and equilibrated for 60 min before the beginning of the experiments. Throughout the experiment, the temperature was kept constant at 37°C and the tissue bath was continuously gassed with 96% O₂ and 4% CO₂. The length was adjusted so that resting tension was around 0.3 mN.

The muscle preparations were stimulated in KCl depolarization solution or with PDBu and isotonic quick releases under constant load were applied during isometric steady-state. The load steps were adjusted to 3%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% and 50% of maximal isometric force. The predetermined force was held constant by the controlled motion of the length step generator which followed the contracting bladder with the appropriate velocity for 150 ms. The next release started only after the force had reached the maximal isometric force.

Force was continuously recorded, while force and length at the time of a release were stored on a computer for later analysis. In the length response three phases are observed: an initial elastic recoil, followed by a rapid shortening (lasting about 25-75 ms), and a subsequent phase (around 100 ms) of more steady shortening velocity. Thus, the steady shortening 100 ms after the release was used for analysis, by fitting a tangent to the length record (automatically calculated by software MUSKI).

The relation between shortening velocity and force during isotonic contraction was analyzed using a linearized form of the Hill (Hill 1938) equation:

$$(P + a)(v + b) = (P_0 + a)b$$

where v is the shortening velocity, P is the force during isotonic contraction, P_0 is the maximal isometric steady-state force and a and b are constants. Through mathematical calculation it results in:

$$(P/P_0)/b + a/b = (1-P/P_0)/v$$

With this formula, the data can be plotted on a diagram and further interpreted by linear regression, where X-axis is P/P_0 , Y-axis is $(1-P/P_0)/v$, slope is $1/b$ and y-intercept is a/b .

The maximal shortening velocity (V_{\max}) was calculated as $(1-P/P_0)/v$ when $P/P_0 = 0$. The shortening velocity was given as muscle length per second (ML/s).

3.5 Statistics

Values are expressed as means \pm S.E.M (n = number of experiments). Differences between means were evaluated using Student's t-test.

RESULTS

4.1 Expression of SM-MHC and NM-MHC isoforms

The presence of smooth muscle myosin heavy chain (SM-MHC) and non-muscle myosin heavy chain (NM-MHC) isoforms in urinary bladders from wild-type (WT) and homozygous SM-MHC knockout (KO) animals was evaluated by Western blot analysis. Western blot analysis using a polyclonal antibody raised against chicken-gizzard SM-MHC showed no SM-MHC signal in KO bladders (Fig. 4). The expression of NM-MHC was further evaluated by Western blot analysis using a specific antibody raised against the rod-portion of NM-MHC, which detected both NMA and NMB isoforms. The NM-MHC signal was verified in bladders from both wild-type and SM-MHC deficient mice (Fig. 5).

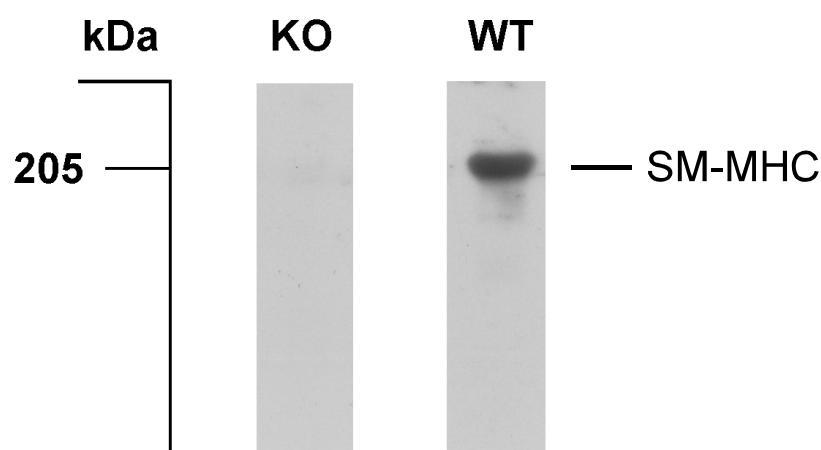


Figure 4. Western blot analysis of SM-MHC expression in bladder from wild-type (WT) and homozygous SM-MHC knockout (KO) mice with a polyclonal antibody raised against chicken-gizzard SM-MHC (1:100000, 20 μ g of protein loaded on each lane), showing the absence of any SM-MHC signal in knockout mice.

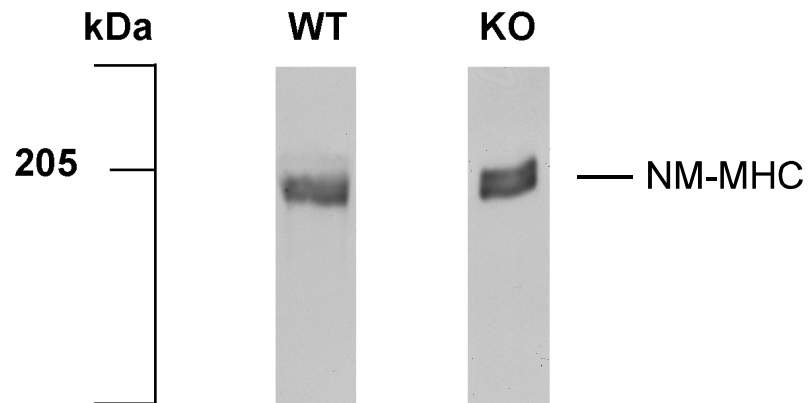


Figure 5. Western blot analysis of NM-MHC expression of wild-type (WT) and homozygous SM-MHC knockout (KO) bladders, using an antibody raised against the rod portion of NM-MHC (1:1250, 20 μ g of protein loaded on each lane), revealing similar expression in both WT and KO bladders.

4.2 Analysis of myosin filamentous structures in smooth muscle cells

Since the formation of filamentous structures of myosin is considered to be a prerequisite for muscle contraction, NM-MHC structures were investigated in primary cultures of bladder cells by immunofluorescence microscopy. A double labeling method was used with a specific antibody raised against the rod-portion of NM-MHC (aNM-MHC) and an antibody against smooth muscle α -actin (aS-actin), as a smooth muscle cell marker. The primary antibodies were visualized with an anti-rabbit and an anti-mouse secondary antibodies, labelled with DTAF (green) and Cy3 (red) respectively. As seen in figure 6, smooth muscle cells prepared from the bladders of both wild-type (WT) and homozygous SM-MHC knockout (KO) mice showed longitudinally arranged intracellular NM-MHC filamentous structures.

In another set of experiments, immunolabeling of smooth muscle cells from the bladders of both wild-type and knockout mice was performed using an antibody raised against chicken gizzard SM-MHC (aSM-MHC) and the aS-actin antibody. The primary antibodies were visualized with an anti-rabbit and an anti-mouse secondary antibodies as described above. As can be seen in figure 7, longitudinally arranged intracellular filamentous structures of SM-MHC were observed in smooth muscle cells prepared from wild-type mice, but not in smooth muscle cells from knockout mice. The smooth muscle cells were identified by reaction with the aS-actin antibody.

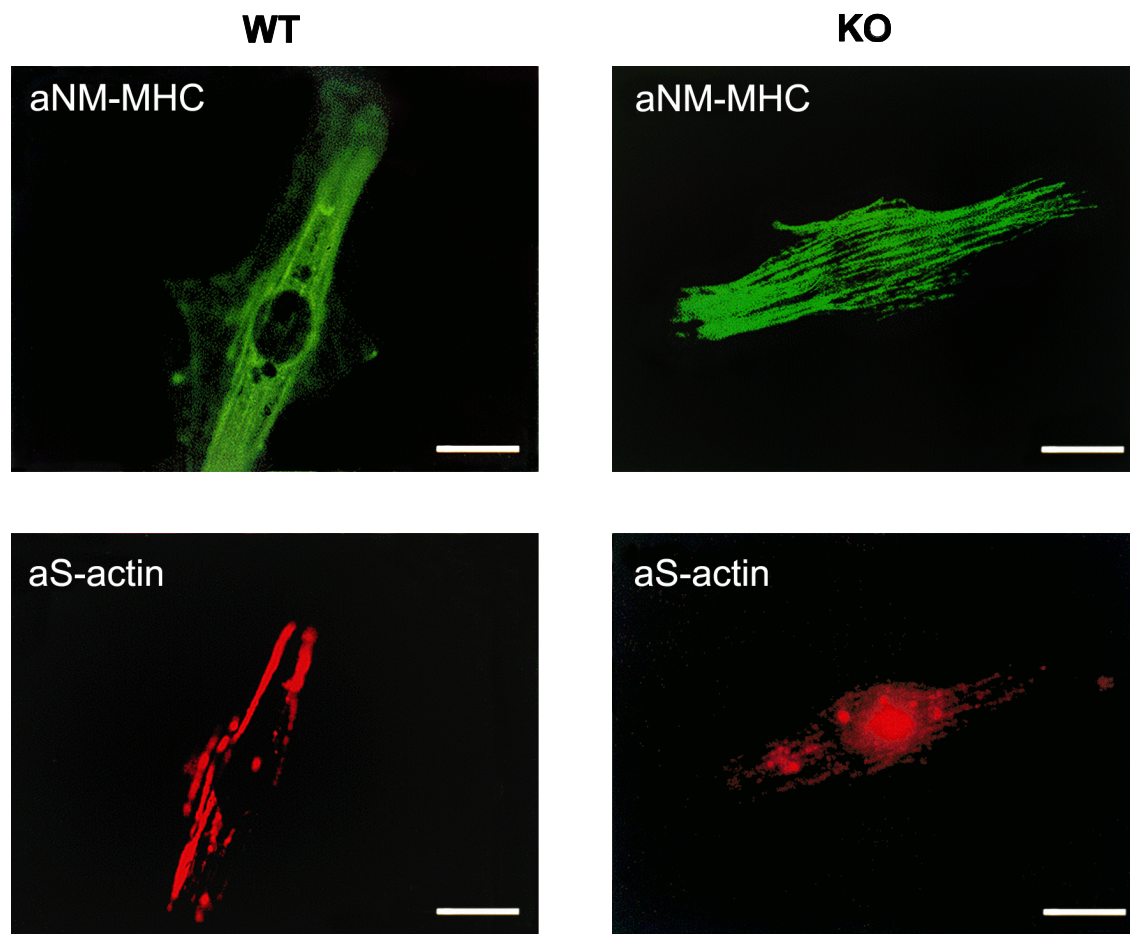


Figure 6. Immunofluorescence micrographs of cultivated smooth muscle cells obtained from the bladders of wild-type (WT; left panels) and homozygous SM-MHC deficient (KO; right panels) mice. Immunoreactions were carried out using an anti-non-muscle myosin heavy chain (aNM-MHC; upper panels, green) and an anti-smooth muscle α -actin (aS-actin; lower panels, red) antibodies. Longitudinally arranged intracellular filamentous structures of NM-MHC were observed in smooth muscle cells from both wild-type and SM-MHC deficient mice. Scale bars represent 5 μ m.

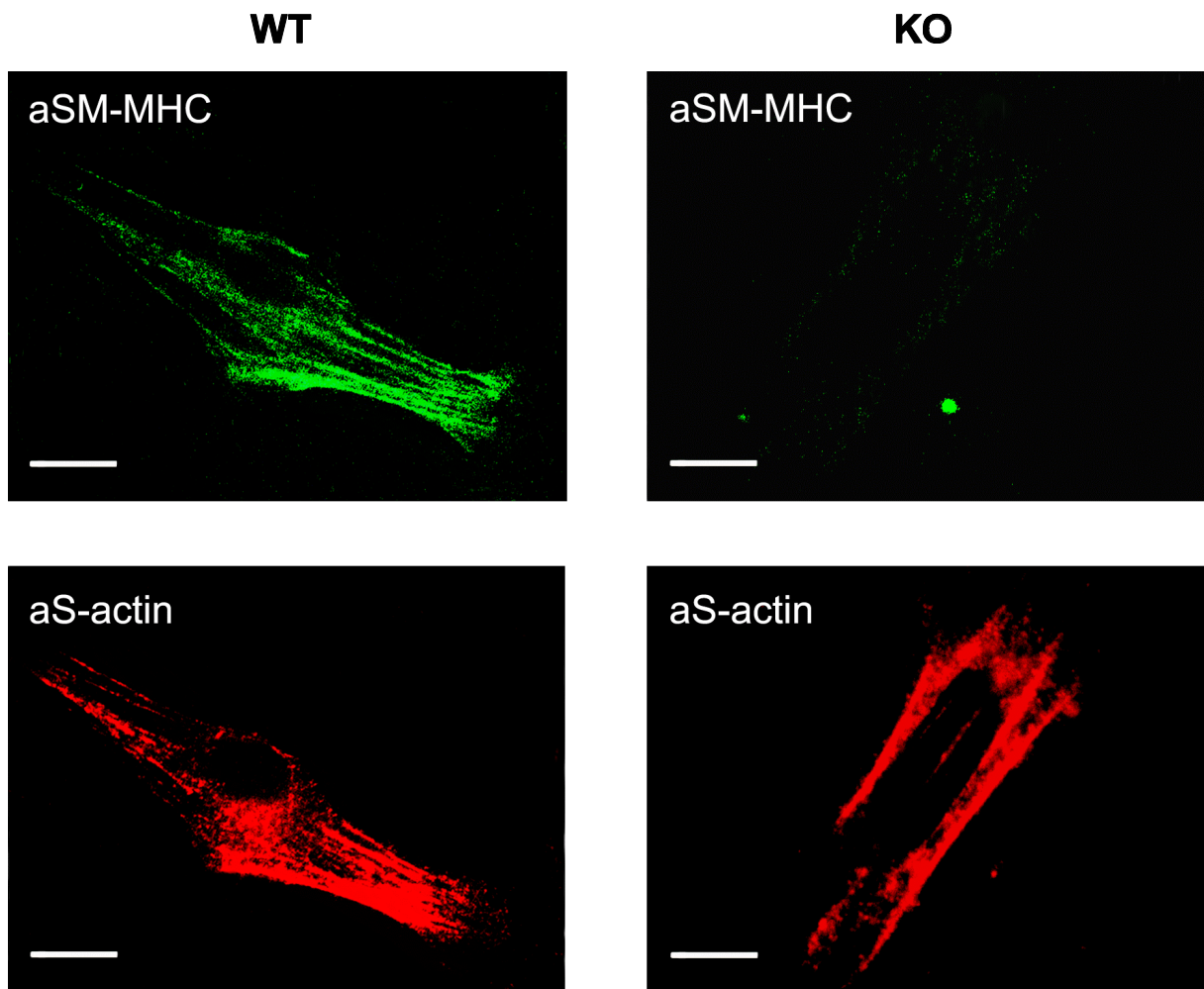


Figure 7. Immunofluorescence micrographs of cultivated smooth muscle cells obtained from the bladders of wild-type (WT; left panels) and homozygous SM-MHC deficient (KO; right panels) mice. Immunolabeling was carried out using an antibody raised against chicken gizzard smooth muscle myosin heavy chain (aSM-MHC; upper panels, green) and an anti-smooth muscle α -actin antibody (aS-actin; lower panels, red). Smooth muscle cells from wild-type mice showed longitudinally arranged intracellular filamentous SM-MHC structures. In smooth muscle cells from knockout mice, however, no SM-MHC signal could be observed. Scale bars represent 5 μ m.

4.3 Mechanical analysis of smooth muscle preparations

Urinary bladder intact strips prepared from neonatal wild-type and SM-MHC deficient mice were used for the mechanical analysis of smooth muscle. The bladder preparations were equilibrated in Krebs-Henseleit solution and then stimulated using prolonged KCl depolarization (50mM KCl substituted for Na⁺ in Krebs-Henseleit solution) or with phorbol 12, 13-Dibutyrate (PDBu).

4.3.1 KCl-induced contraction

4.3.1.1 Isometric contraction of wild-type bladder preparations

Using KCl depolarization of intact bladder preparation from neonatal wild-type mice, an initial transient state (phase 1) and a subsequent sustained state (phase 2) of isometric force generation could be distinguished (Fig. 8). Force at the peak of the initial transient phase was 2.93 ± 0.37 mN *i.e.* significantly higher than the subsequent sustained force (0.79 ± 0.19 mN) ($p < 0.001$, $n = 8$ animals) (Fig. 10).

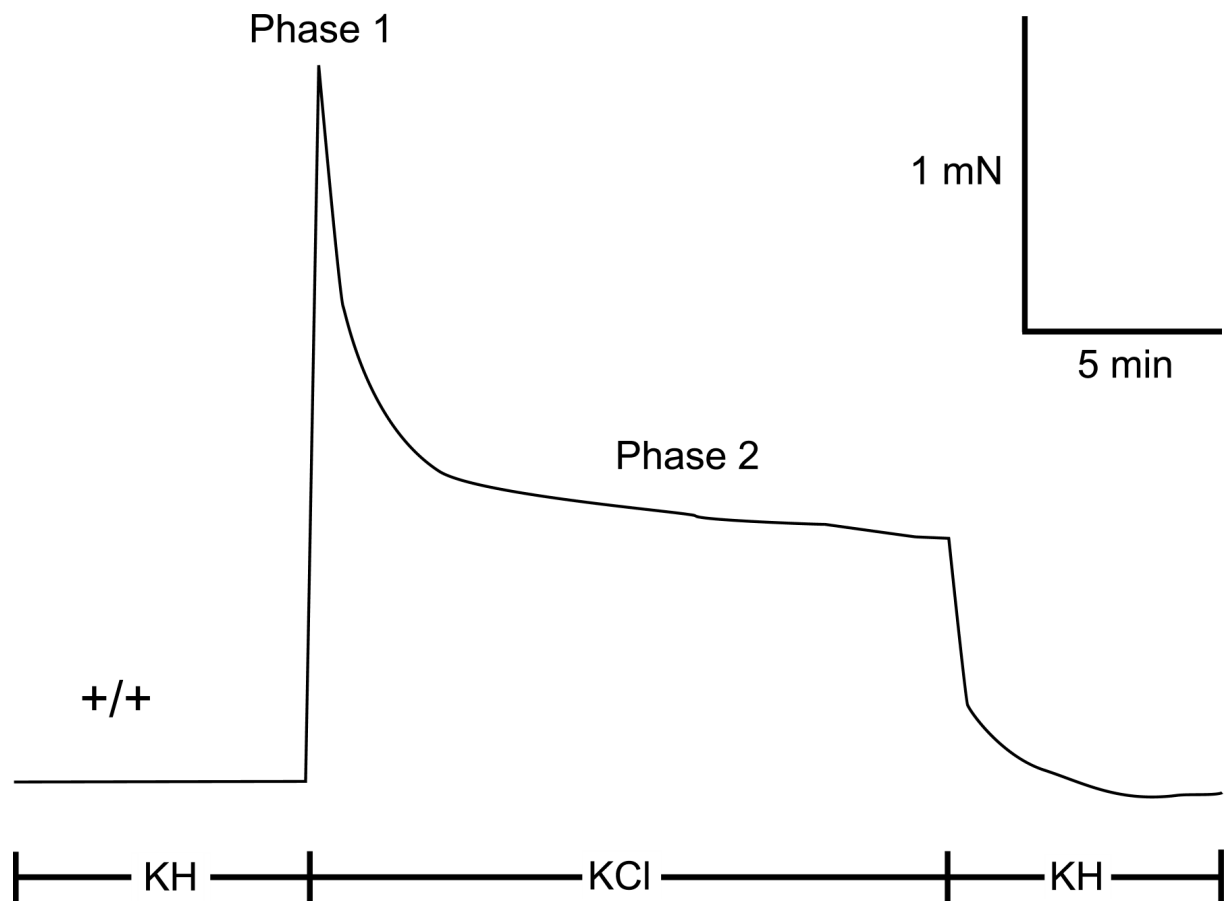


Figure 8. Original recording of isometric force in bladder preparation of wild-type (+/+) mouse. KH, Krebs-Henseleit solution (relaxation solution); KCl, depolarization solution (contraction solution).

4.3.1.2 Isometric contraction of knockout bladder preparations

KCl depolarization of intact bladder preparations from SM-MHC deficient mice induced only a sustained phase (phase 2) *i.e.* without the initial transient high-force state (Fig. 9). Sustained contraction of bladder preparations from SM-MHC deficient mice was 0.59 ± 0.07 mN ($n = 8$ animals) *i.e.* not significantly different compared with the sustained force of wild-type preparations, but significantly lower than the initial transient force of wild-type preparations ($p < 0.001$) (Fig. 10).

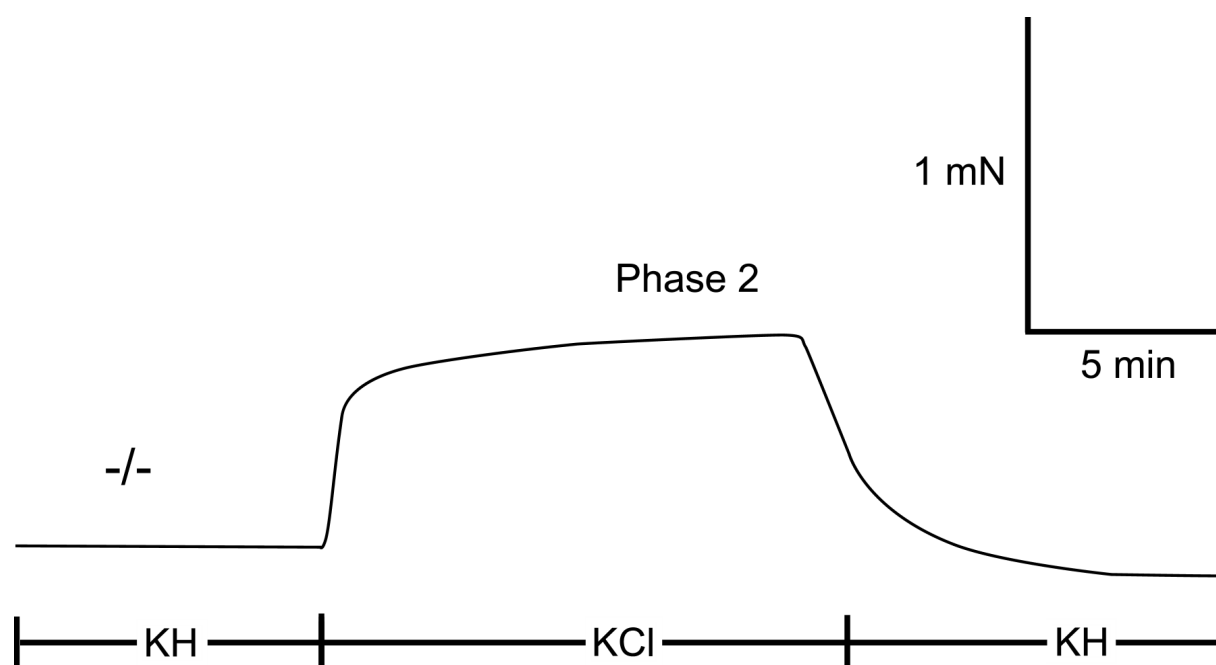


Figure 9. Original recording of isometric force in bladder preparation of homozygous SM-MHC deficient (-/-) mouse. KH, Krebs-Henseleit solution (relaxation solution); KCl, depolarization solution (contraction solution).

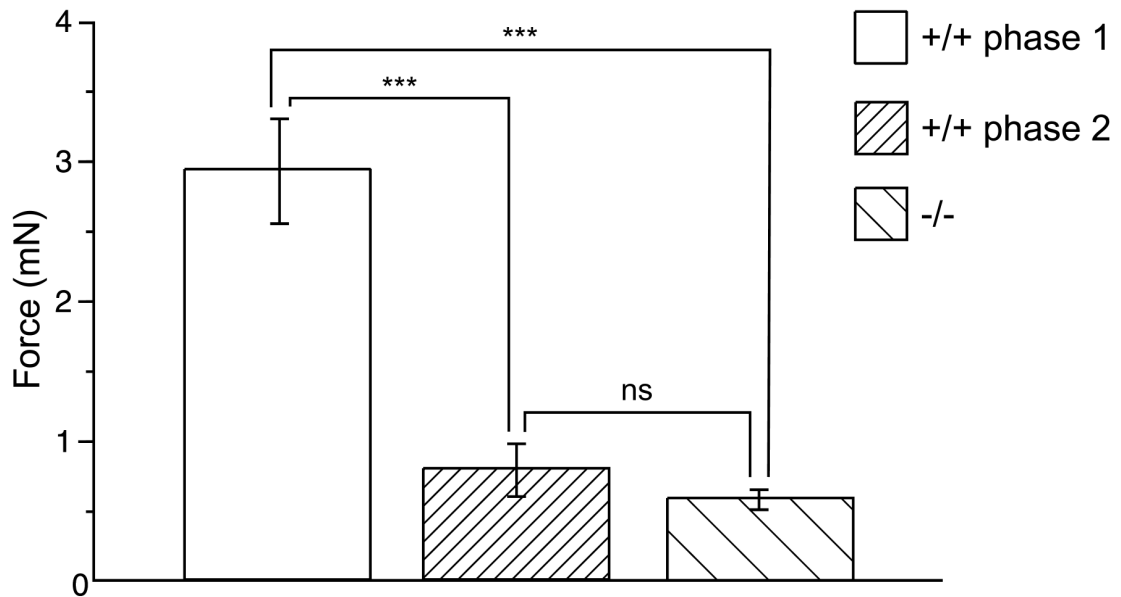


Figure 10. Statistical comparison of isometric force generated in phase 1 (around 1 min after onset of KCl stimulation) and phase 2 (around 15 min after KCl stimulation) of smooth muscle preparations of wild-type (+/+) and phase 2 of homozygous SM-MHC deficient (-/-) mice. *** denotes $p < 0.001$; ns, no significance; values are mean \pm S.E.M.

4.3.1.3 Isotonic contraction of wild-type bladder preparations

Bladder preparations from wild-type (WT) mice revealed a maximal shortening velocity (V_{max}) at the peak of phase 1 (initial high force generation) of 0.41 ± 0.03 muscle length/second (ML/s). V_{max} decreased significantly ($p < 0.001$) 15 min after the onset of KCl stimulation (phase 2) to 0.15 ± 0.01 ML/s ($n = 9$ animals) (Fig. 12).

4.3.1.4 Isotonic contraction of knockout bladder preparations

In similar preparations from homozygous SM-MHC knockout mice (KO), V_{max} measured 15 min after onset of KCl stimulation (phase 2) was 0.16 ± 0.02 ML/s, *i.e.* similar to the corresponding phase of contraction in wild-type mice, but significantly lower ($p < 0.001$) than the V_{max} of the initial phase of contraction from wild-type preparations. The V_{max} of knockout bladder preparations determined 1 min after onset of KCl stimulation (*i.e.* prior reaching steady-state force) was 0.16 ± 0.02 ML/s *i.e.* not different from the values obtained 15 min after KCl stimulation ($n = 7$ animals) (Fig. 12).

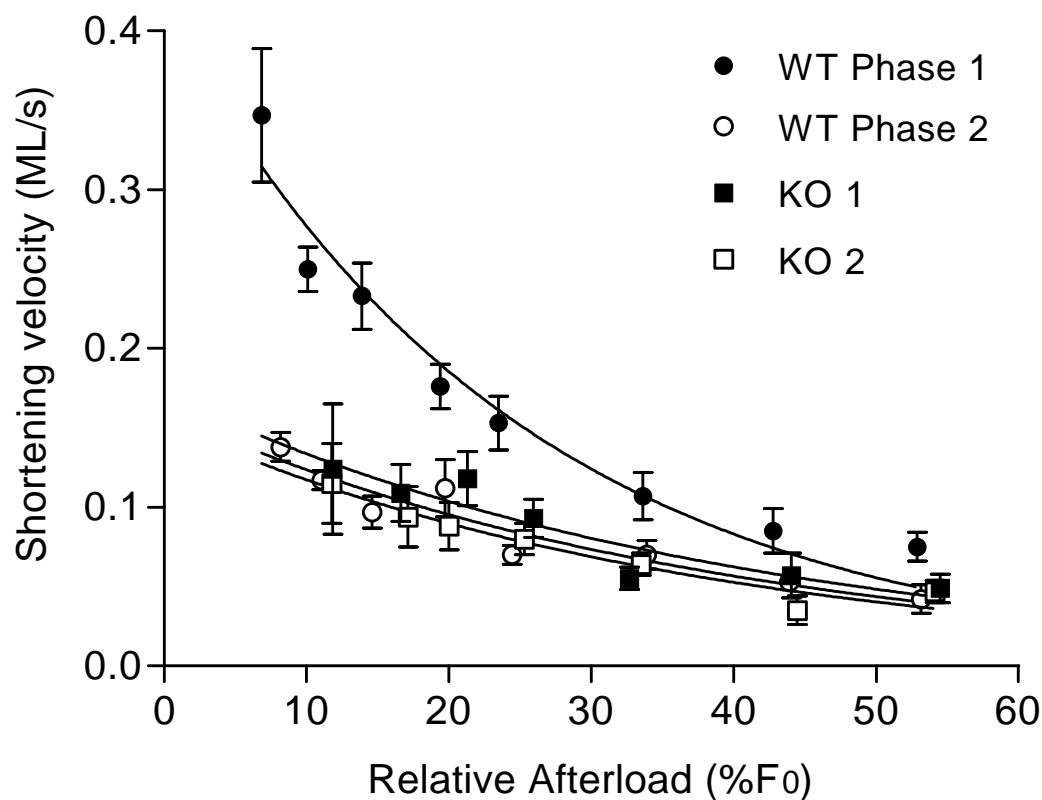


Figure 11. Force-velocity relationship of intact KCl-stimulated bladder preparations obtained during the peak of the initial transient high-force phase (phase 1; cf. 8) and 15 min after onset of KCl depolarization during the subsequent sustained phase (phase 2; cf. 8) of wild-type (WT) mice, as well as 1 min (KO 1) and 15 min (KO 2; phase 2, cf. 9) after KCl depolarization of preparations from knockout (KO) mice. ML/s, muscle length per second. %F₀, force during the quick release expressed in % of maximal isometric force.

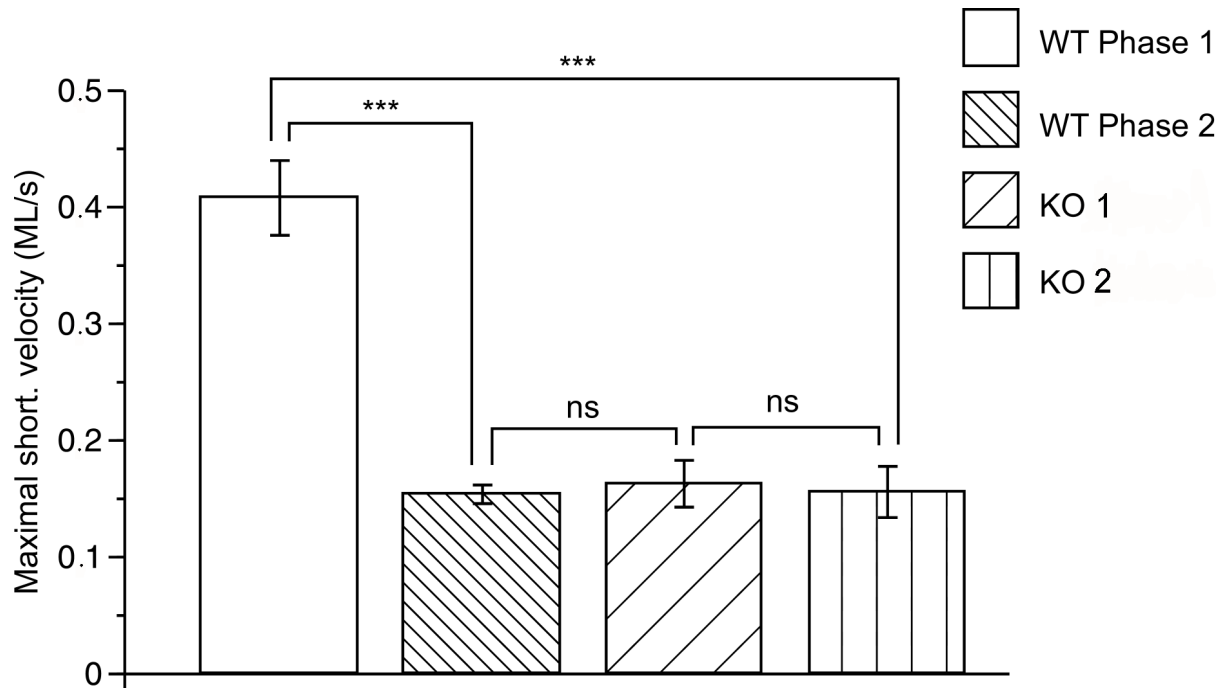


Figure 12. Statistical comparison of maximal shortening velocity of phase 1 and phase 2 of wild-type (WT) and phase 2 (KO 1, after 1 min; KO 2, after 15 min stimulation) of SM-MHC knockout preparations. ML/s, muscle length per second. *** denotes $p < 0.001$; ns, no significance; values are mean \pm S.E.M.

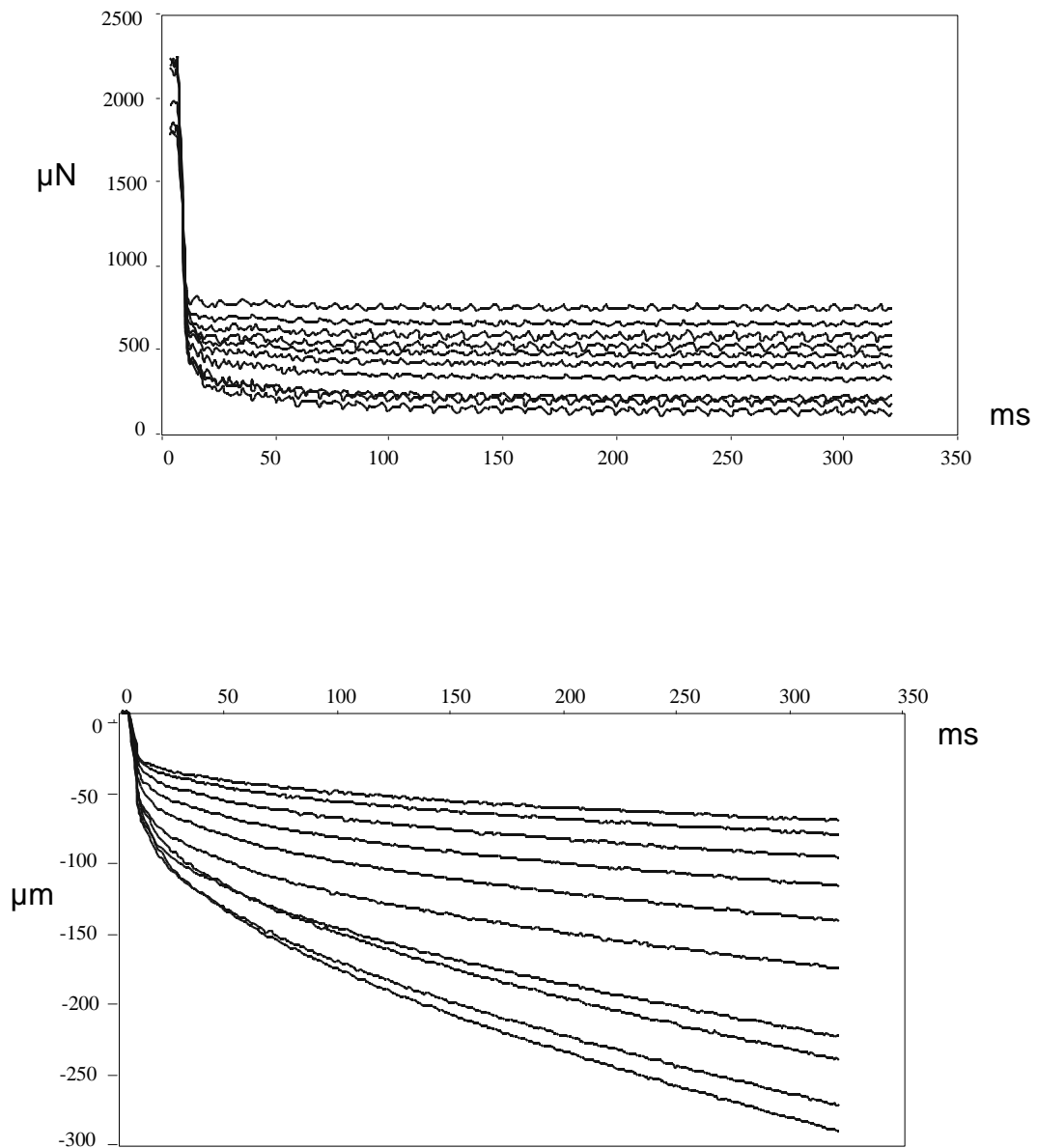


Figure 13. Original recordings of isotonic quick release measurements in intact smooth muscle preparations. Upper traces, force (μN). Lower traces, the corresponding length (μm). X axis, time (ms). Shortening velocity is the slope of the curves.

4.3.2 PDBu-induced contraction

4.3.2.1 Isometric contraction of wild-type bladder preparations

Concentration-response curves for PDBu were made using intact bladder preparations from neonatal wild-type mice, in order to determine the adequate concentration for use. The concentration-response curves were performed accumulatively, after the response to the previous concentration had reached a plateau. PDBu (10^{-8} - 10^{-5} M) was shown to induce concentration-dependent increased isometric contractions in intact bladder preparations from neonatal wild-type mice, with half-maximal concentration of $0.56 \mu\text{M}$, and maximal concentration of $10 \mu\text{M}$. The contractile response to PDBu (10^{-5} M) was 0.26 ± 0.03 mN in wild-type smooth muscle preparations ($n = 8$ animals).

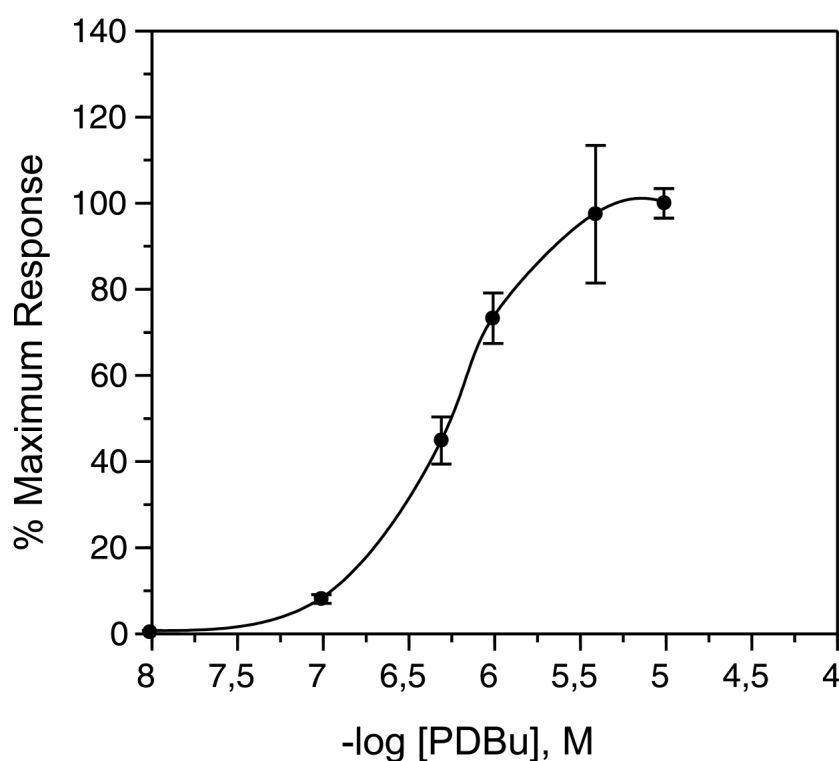


Figure 14. Concentration-response curve for PDBu in urinary bladder smooth muscle from neonatal wild-type mice. Each data point represents the mean \pm S.E.M. of 5 experiments. Force is expressed as a percentage of the maximal response.

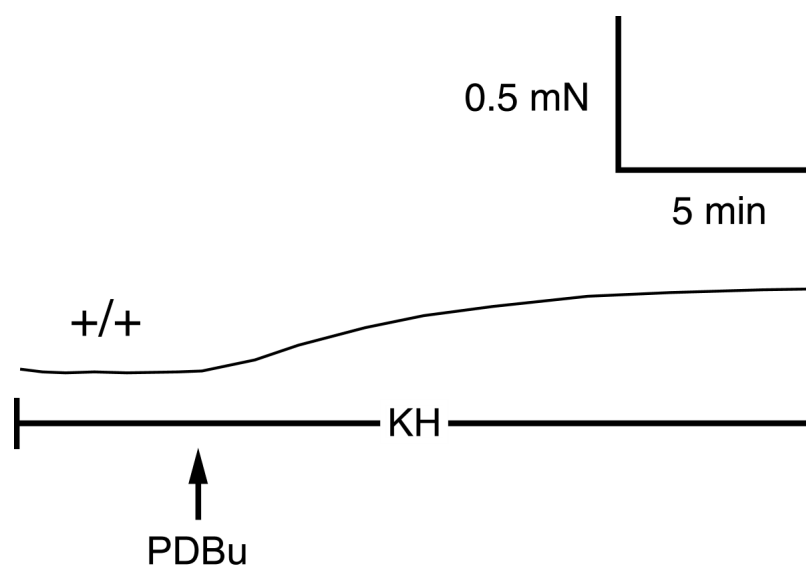


Figure 15. Original recording of isometric force induced by PDBu in intact bladder preparation of wild-type (+/+) mouse. The arrow indicates the addition of PDBu (10^{-5} M) in the tissue bath. KH, Krebs-Henseleit solution (relaxation solution).

4.3.2.2 Isometric contraction of knockout bladder preparations

Bladder preparations from SM-MHC deficient mice (-/-) showed a sustained contraction after PDBu (10^{-5} M) stimulation, similar to those of wild-type mice (Fig. 16). The contractile response to PDBu was 0.39 ± 0.07 mN ($n = 8$ animals). This value was not significantly different from PDBu-induced contraction of wild-type preparations (Fig. 17).

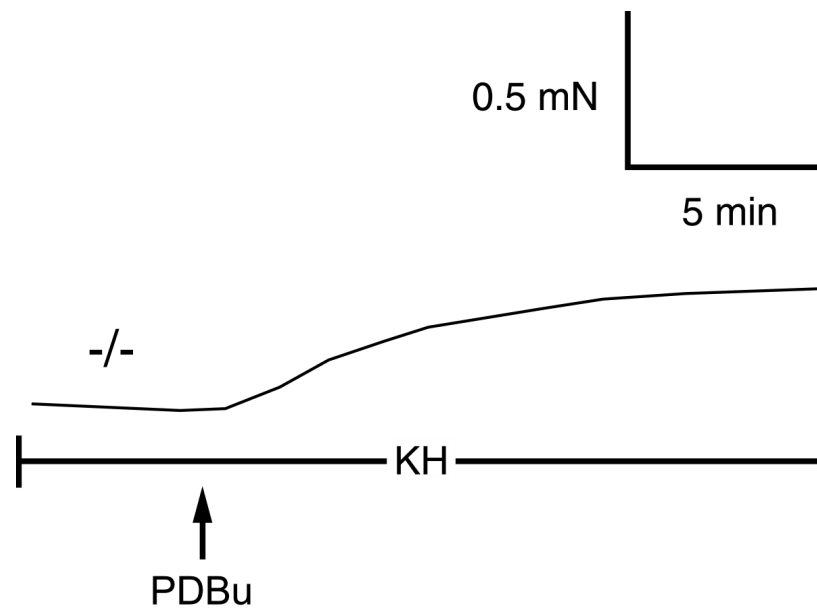


Figure 16. Original recording of isometric force induced by PDBu in intact bladder preparation of SM-MHC deficient (-/-) mice. The arrow indicates the addition of PDBu (10^{-5} M) in the tissue bath. KH, Krebs-Henseleit solution (relaxation solution).

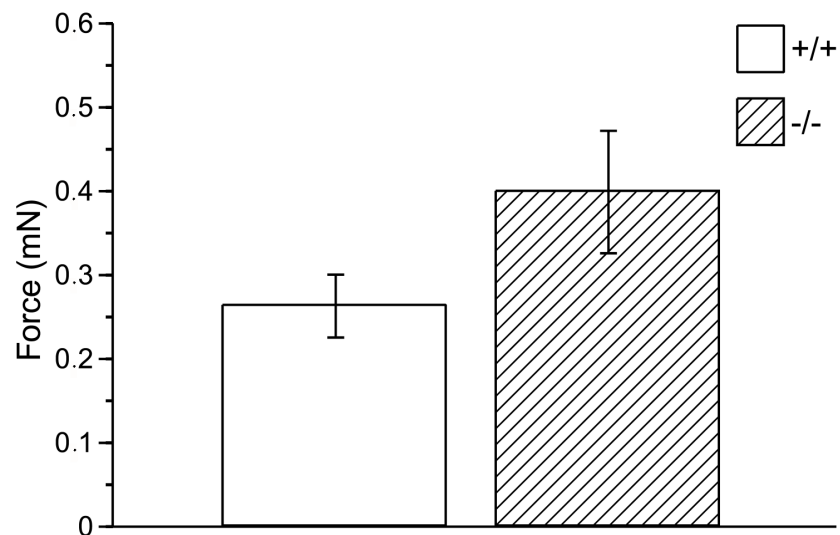


Figure 17. Statistical comparison of isometric force generated upon PDBu stimulation of bladder preparations of wild-type (+/+) and SM-MHC knockout (-/-) mice. Means are not significantly different, values are mean \pm S.E.M. ($n = 8$ animals per group).

Incubation with the PKC inhibitor, Ro-31-7549, completely abolished the force generated upon PDBu stimulation in both smooth muscle preparations from wild-type (+/+) and SM-MHC deficient mice (-/-) (Fig. 18).

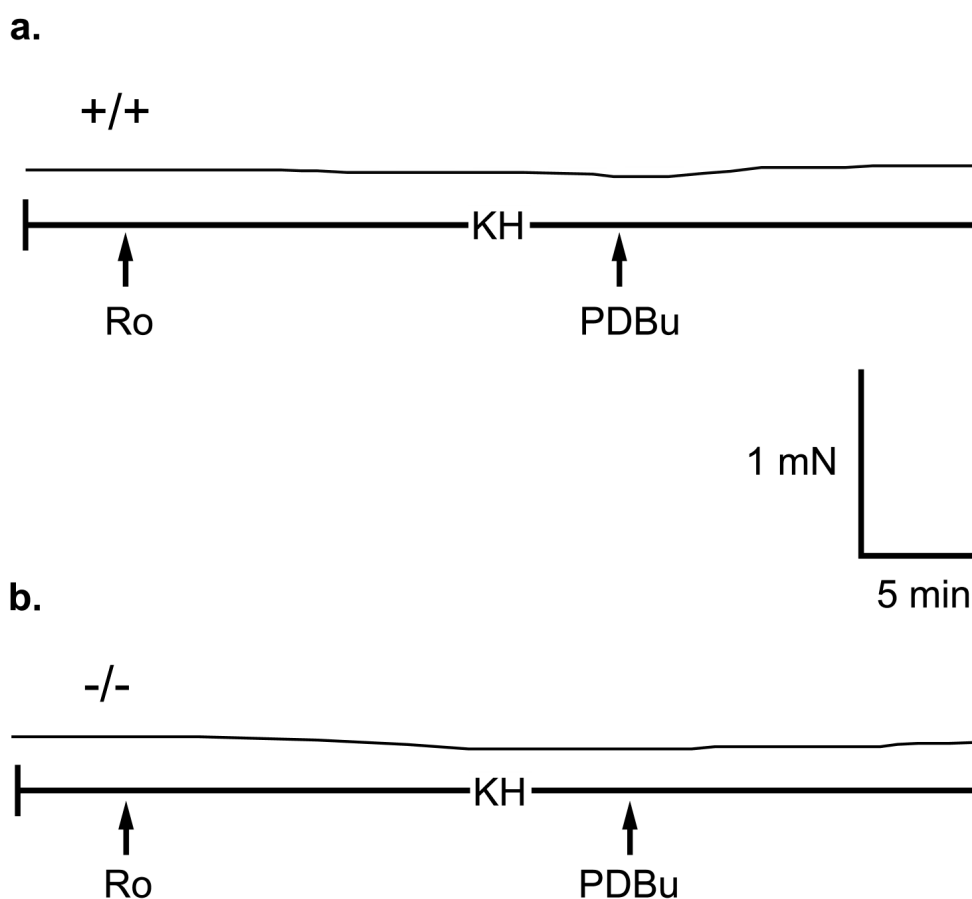


Figure 18. Original recordings of isometric force induced by PDBu in presence of the cell permeable PKC inhibitor (Ro-31-7549, 5 μ M). PDBu (4 μ M) was applied into the tissue bath containing Krebs-Henseleit solution. **a**, smooth muscle preparation of wild-type (+/+) mouse. **b**, smooth muscle preparation of homozygous SM-MHC deficient (-/-) mouse. KH, Krebs-Henseleit solution (relaxation solution).

4.3.2.3 Effect of PDBu on KCl-induced contraction

The effect of PDBu on both phases of KCl-induced contraction was examined in intact bladder preparations from neonatal wild-type mice. Pre-incubation with PDBu significantly potentiated the sustained phase of KCl-induced contraction, but had nearly no effect on the initial phase of contraction (Fig. 19). The effect was expressed as percentage of isometric force elicited by KCl before incubation with PDBu (force after PDBu/force before PDBu · 100). Sustained force (phase 2) was $190 \pm 24.5\%$ of isometric force before PDBu, whereas the transient phase (phase 1) of KCl-induced contraction increased only to $110.2 \pm 2.5\%$ ($p < 0.01$, $n = 6$ animals per group) upon treatment with PDBu (Fig. 20).

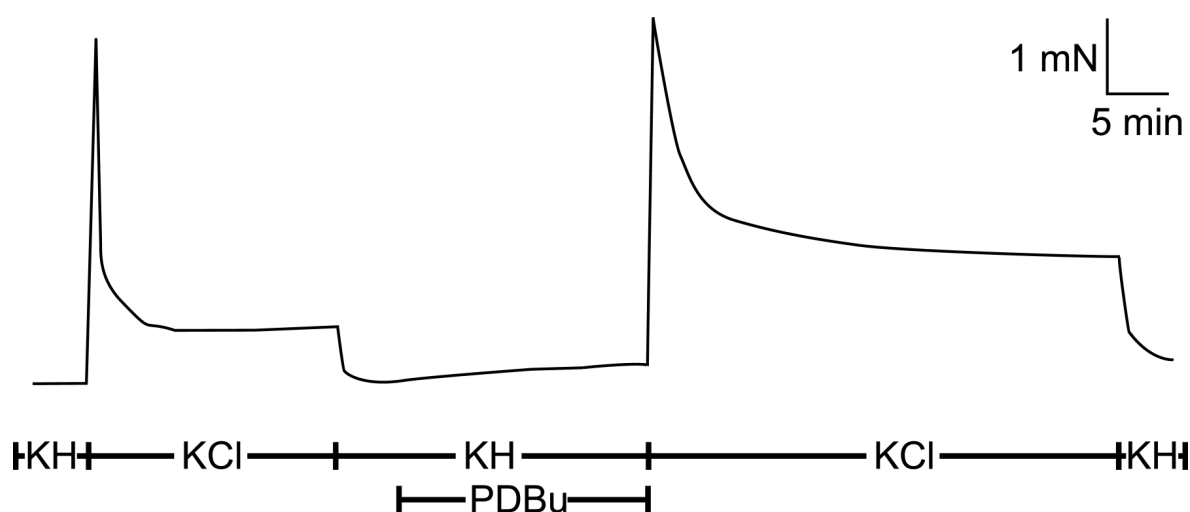


Figure 19. Original recording of isometric force induced by KCl depolarization after pre-incubation with PDBu in intact smooth muscle preparation of wild-type mouse. KH, Krebs-Henseleit solution (relaxation solution); KCl, depolarization solution (contraction solution).

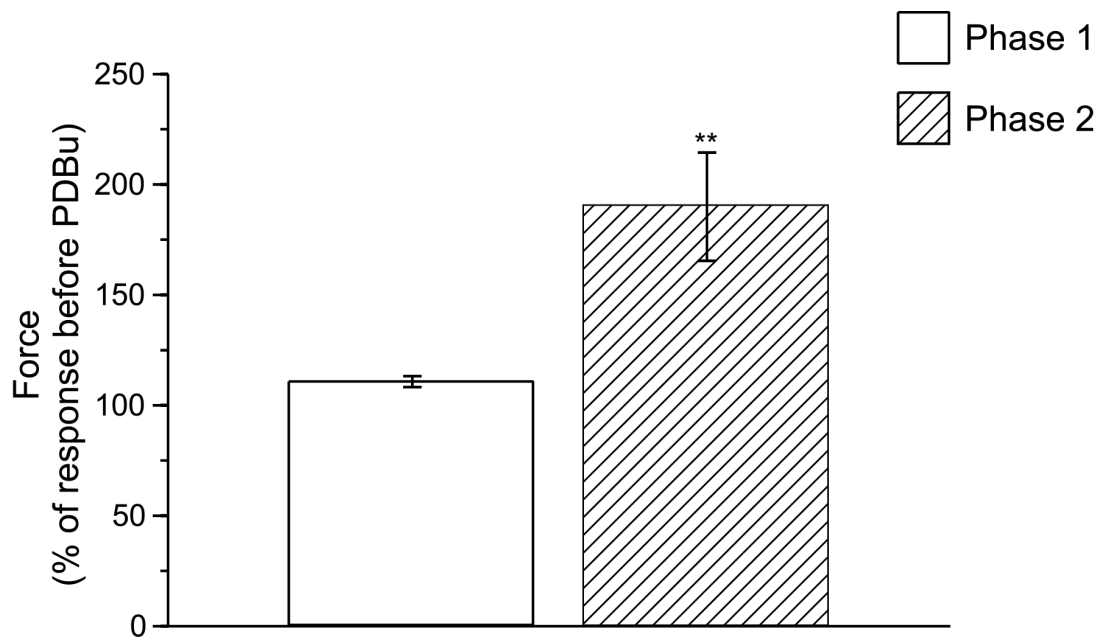


Figure 20. Statistical comparison of the effect of pre-treatment with PDBu on initial (phase 1) and sustained (phase 2) phases of KCl-induced contraction, expressed as percentage of isometric force elicited before PDBu. ** denotes $p < 0.01$; values are mean \pm S.E.M. ($n = 6$ animals per group).

DISCUSSION

The main finding of the present study is that non-muscle myosin heavy chains are recruited for smooth muscle contraction. This was verified by the observation that smooth muscle preparations without smooth muscle myosin heavy chain (SM-MHC) expression, but with normal non-muscle myosin heavy chain (NM-MHC) expression, produced sustained force of contraction. This force generation is elicited by myosin II, as demonstrated by cross-bridge cycling (active shortening) of all smooth muscle preparations in the constant load experiment.

In gene targeted mice, a total depletion of SM-MHC was observed, as demonstrated by Western blot analysis using an antibody against chicken-gizzard SM-MHC. Additionally, the absence of SM-MHC was recently confirmed by both Northern blot and Western blot analysis using antibodies against the isoforms SM1, SM2 and the seven-amino-acid insert of SM-MHC (Morano et al. 2000). Ablation of the SM-MHC gene, however, did not affect the expression of non-muscle myosin heavy chain. This was demonstrated by Western blot analysis with a specific antibody against the rod-portion of NM-MHC, showing that NM-MHC was normally expressed in SM-MHC deficient mice.

The involvement of NM-MHC in smooth muscle contraction was supported by the existence of longitudinally arranged NM-MHC filaments in smooth muscle cells from both wild-type and knockout mice, using immunofluorescence microscopy. The myosin filaments serve as the basic functional units necessary for contraction. As expected, smooth muscle cells from knockout mice showed no SM-MHC filaments. Additionally, the presence of longitudinally arranged filamentous structures of SM-MHC in wild-type smooth muscle cells confirmed that the cultivation conditions (24h) used in the primary culture of smooth muscle cells conserved the contractile phenotype.

The mechanical analysis showed that smooth muscle preparations from SM-MHC deficient mice produced force and active shortening. The force development in the muscle is correlated with the number of activated cross-bridges, whereas shortening manifests the cross-bridge turnover rate. Both mechanical and kinetic properties from the muscle

reflect the actin-myosin interaction. Thus, the force generated by knockout preparations could only be produced by recruitment of myosin II rather than by changes in the compliance of elastic elements. The mechanical experiments were realized with intact muscle preparations, because using this method physiological conditions are preserved. Therefore, it was possible to verify the physiological significance of non-muscle myosin *in vivo*.

Non-muscle myosin II is a ubiquitous cytoskeletal protein, with high homology to smooth muscle myosin II. In non-muscle cells, it has an important role in various motile events, like cell division (De Lozanne and Spudich 1987; Fukui et al. 1990), cell motility (Warrick and Spudich 1987) and cell morphology (Pasternak et al. 1989). NM-MHC is also expressed in smooth muscle cells under normal physiological conditions and during pathological processes (Kelley 1997). Moreover, the concentration of NM-MHC in smooth muscle cells is 2- to 3-fold greater than that of platelets and adrenal medulla (Gaylinn et al. 1989). Because of this large quantity of protein, it would be expected that it has some physiological significance. However, its function in smooth muscle cells is still not completely clear. NM-MHC expression increases during smooth muscle cell proliferation and atherosclerotic processes (Kawamoto and Adelstein 1987; Kuro-o et al. 1991; Simons et al. 1993; Simons and Rosenberg 1992). Thus, it has been proposed that non-muscle myosin is involved in the proliferative phenotype of smooth muscle cells. However, there was no evidence about the participation of NM-MHC in the contractile phenotype of smooth muscle cells.

A preferential localization of non-muscle myosin in the cytoskeletal domain has been suggested, proposing that this isoform may not be involved in smooth muscle contraction (Murphy et al. 1997). This hypothesis of intracellular compartmentation of myosin isoforms is mainly supported by a study showing a primarily localization of non-muscle actin isoform in the dense bodies and near the intermediate filaments, while smooth muscle specific actin isoform was localized surrounding the thick filaments (North et al. 1994a). Since the expression of non-muscle myosin and actin are thought to be coordinated (Gaylinn et al. 1989), the evidence of actin isoforms compartments in smooth muscle cells would suggest a parallel localization of non-muscle myosin isoforms in the cytoskeletal domain. However, a study with isolated native thin filaments showed that

different isoactin monomers were present in an individual thin filament (Drew et al. 1991), thus arguing against the intracellular compartmentation of actin isoforms, and consequently of myosin isoforms.

According to the above studies, it is generally believed that the non-muscle myosin isoforms respond to cellular functions, like proliferation and migration, but have no participation in the contractile function of smooth muscle cells. Nevertheless, most information about the function of non-muscle and smooth muscle myosin isoforms has come from *in vitro* studies. In the present study, the experimental animal model used allowed to specifically investigate the function of NM-MHC *in vivo*. Thus, for the first time the participation of non-muscle myosin in the contractile function of smooth muscle cells under physiological conditions was shown.

The second important finding of this study is that smooth muscle cells from neonatal mice could switch from one myosin isoform to another during prolonged stimulation. The smooth muscle preparations from wild-type mice showed an initial phase of high force generation (phase 1), followed by a sustained phase of contraction (phase 2). Similar preparations from SM-MHC deficient mice, however, showed only a sustained contraction, without a phase 1. In addition, the muscle preparations from knockout mice showed similar contractile (isometric force) and kinetic (shortening velocity) features as the phase 2 of wild-type preparations. Thus, it seems that SM-MHC is recruited for the phase 1, whereas NM-MHC is used subsequently for force maintenance (phase 2). The involvement of NM-MHC in phase 2 of normal neonatal bladder is reinforced by the existence of longitudinally arranged NM-MHC filaments in wild-type smooth muscle cells by immunofluorescence microscopy, similar to the NM-MHC filamentous structures observed in the cell cultures from knockout mice. In fact, using the *Nitella*-based *in vitro* motility assay, Umemoto et al. showed that NM-MHC from human platelets moved actin filaments much slower than SM-MHC from aorta or trachea bovine and turkey gizzard smooth muscle (Umemoto et al. 1989). This difference observed in the movement of smooth muscle and non-muscle (platelets) myosin could explain the lower V_{max} of phase 2, in comparison with phase 1 of contraction, thus supporting our hypothesis of involvement of NM-MHC in phase 2 of wild-type contraction.

Some studies have shown that shortening velocity or ATPase activity can be modified

during the course of a contraction. Initially, the increase in intracellular Ca^{2+} leads to MLC_{20} phosphorylation and tension generation. During this phase, myosin ATPase activity is high, as well as ATP utilization and oxygen consumption. Subsequently, myosin ATPase activity, ATP utilization and oxygen consumption fall, but tension is maintained (Summerville and Hartshorne 1986). Several different regulatory processes have been proposed to explain this characteristic, called “latch state” (Dillon et al. 1981). The low V_{max} obtained in the sustained contraction of wild-type and knockout mice, in the present study, is in accordance with the low ATPase activity and low energy consumption during the “latch state”, since there is a close correlation between maximal shortening velocity and myosin ATPase activity (Bárány 1967).

Besides the low ATPase activity, there is also a decrease in the intracellular levels of Ca^{2+} during the “latch state”. Interestingly, Ca^{2+} -independent phosphorylation of non-muscle myosin and contraction of non-muscle cells (expressing exclusively NM-MHC) and Ca^{2+} -sensitization of smooth muscle cells (expressing both SM- and NM-MHC) were recently reported (Kolodney et al. 1999; Somlyo et al. 1999). Therefore, switching to NM-MHC during prolonged smooth muscle activation could serve as an additional mechanism explaining force generation despite decline of Ca^{2+} to almost basal levels during the “latch state”. In fact, both wild-type and SM-MHC deficient neonatal bladder preparations revealed a similar intracellular Ca^{2+} transient, i.e. high, initial intracellular Ca^{2+} peak, which dropped to almost basal levels during sustained contraction (Arner, personal communication).

Another important finding of this study is the involvement of protein kinase C (PKC) in the recruitment of non-muscle myosin. Smooth muscle preparations from both wild-type and knockout mice showed a similar sustained contraction upon stimulation with phorbol ester. Thus, during phorbol ester stimulation, NM-MHC may be recruited in both wild-type and knockout preparations.

Tumor-promoting phorbol esters have been shown to directly activate PKC, probably due to its analogous structure to diacylglycerol (Castagna et al. 1982; Niedel et al. 1983). In the present study, pre-incubation with a specific protein kinase C inhibitor completely abolished the force generated upon stimulation with phorbol ester in both wild-type and knockout muscle preparations, thus confirming the activation of PKC as being responsible

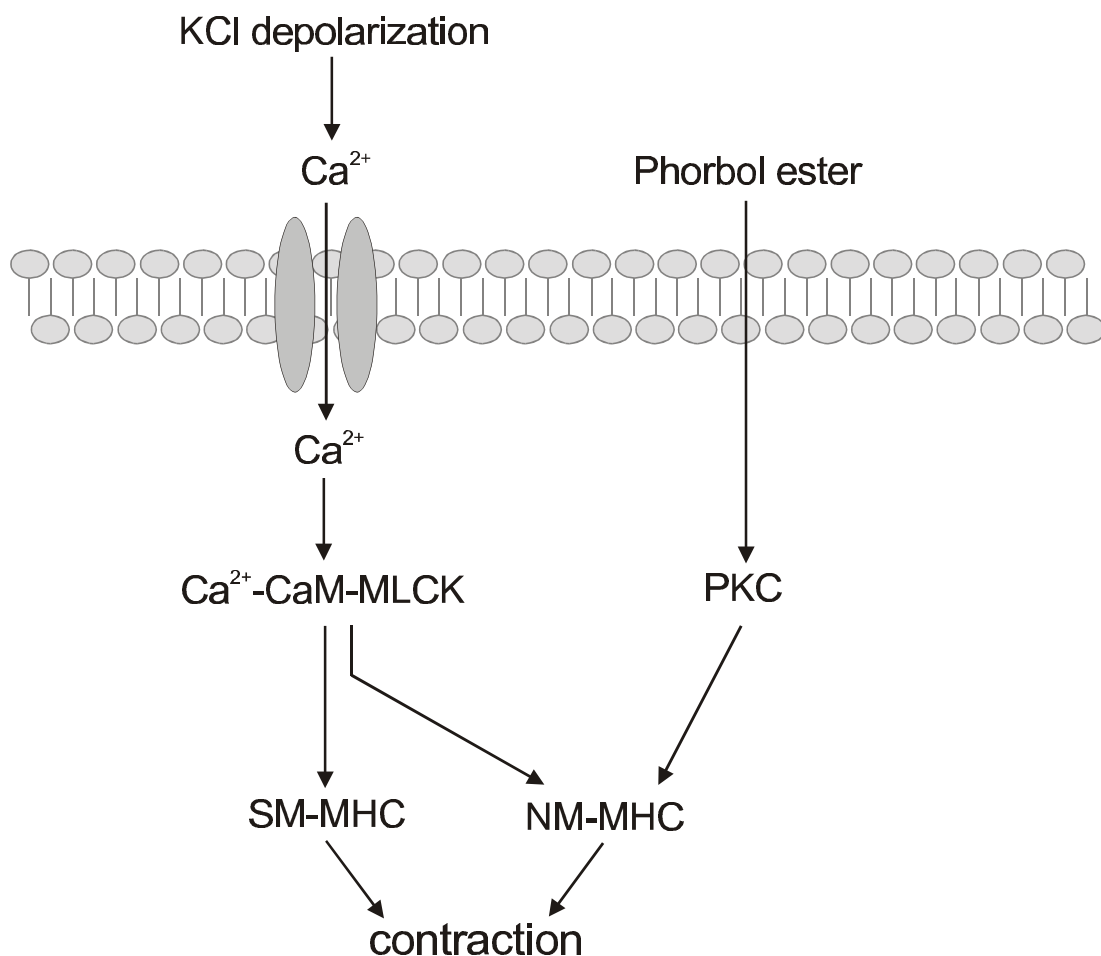
for the force produced by phorbol ester stimulation.

Non-muscle myosin II, similarly to smooth muscle myosin, is primarily activated through phosphorylation of the regulatory light chains of myosin (MLC₂₀). Phosphorylation of non-muscle and smooth muscle MLC₂₀ at Ser19 regulates its ATPase activity and the assembly of myosin into filaments (Bresnick 1999; Trybus 1996). In smooth muscle, MLC₂₀ phosphorylation is mediated by Ca²⁺-calmodulin-activated myosin light chain kinase (MLCK) (Kamm and Stull 1985). In non-muscle cells, not only Ca²⁺-activation of MLCK (Sellers et al. 1981), but other Ca²⁺-independent pathways are believed to be involved in non-muscle myosin activation (Kolodney et al. 1999). Activation of protein kinase C with phorbol esters has been shown to stimulate phosphorylation of the MLC₂₀ in non-muscle cells in both *in vitro* and *in vivo* (Ikebe and Reardon 1990; Kawamoto et al. 1989; Naka et al. 1983; Strassheim et al. 1999). However, the effects of PKC-induced MLC₂₀ phosphorylation remain unclear, since PKC phosphorylation of MLC₂₀ was not associated with increase of ATPase activity of both smooth muscle and non-muscle myosin in the *in vitro* studies (Ikebe and Reardon 1990; Nishikawa et al. 1984), or with myosin movement in the *Nitella*-based motility assay (Umemoto et al. 1989). On the contrary, another study with embryonic smooth muscle myosin isoform (NMB) showed that PKC phosphorylation induced an increase in myosin ATPase activity (de Lanerolle and Nishikawa 1988). Another possible pathway for PKC-induced activation of non-muscle myosin is through the inhibition of myosin light chain phosphatase (MLCP). As in smooth muscle, inhibition of MLCP activity appears to be an important regulatory mechanism for non-muscle cell contraction (Somlyo and Somlyo 2000). Interestingly, a recent study showed that activation of PKC in human platelet induced phosphorylation of CPI, leading to inhibition of MLCP activity and consequently to increased MLC₂₀ phosphorylation (Watanabe et al. 2001). It has been suggested that platelet CPI is a homologous protein of smooth muscle CPI-17, a phosphorylation-dependent inhibitory protein for myosin light chain phosphatase. Thus, CPI phosphorylation by PKC may participate in the regulation of non-muscle myosin activity.

MLC₂₀ phosphorylation catalysed by Ca²⁺-calmodulin-dependent MLCK activation is the main mechanism for initiating contraction in smooth muscle cells. However, other important mechanisms that modulate Ca²⁺-sensitivity of smooth muscle contraction

appear to be important (Somlyo and Somlyo 1999). Several studies support the participation of PKC in sustained contractions of smooth muscle. Activation of PKC through phorbol ester produces sustained contraction of various types of smooth muscle (Park and Rasmussen, 1985; Rasmussen et al. 1984; Yoshida et al. 1992). In the present study, pre-incubation with phorbol ester significantly potentiated the sustained phase of KCl-induced contraction, whereas a minimal effect was observed in the first phase of contraction.

In conclusion, non-muscle myosin is recruited during prolonged KCl-induced sustained contraction, while SM-MHC is recruited early during initial KCl activation. Additionally, protein kinase C activation through stimulation with phorbol ester recruits non-muscle myosin.



SUMMARY

The aim of the present study was to investigate the involvement of non-muscle myosin heavy chain in smooth muscle contraction under physiological conditions. As an experimental model urinary bladder from neonatal wild-type mice as well as from neonatal mice with disrupted smooth muscle myosin heavy chain expression was used. This animal model was established through gene targeting technology, resulting in complete elimination of the expression of smooth muscle myosin heavy chains. The lack of expression of smooth muscle myosin heavy chains was confirmed by electrophoresis and immunoblotting. On the other hand, non-muscle myosin heavy chain expression remained normal, as verified by Western blot analysis.

The mechanical analysis of smooth muscle was performed with intact urinary bladder preparations, stimulated using prolonged KCl depolarization or with phorbol ester. Prolonged activation by KCl depolarization of intact bladder preparations from wild-type neonatal mice produced an initial transient state (phase 1) of high force generation and maximal shortening velocity, followed by a sustained state (phase 2) with lower force generation and maximal shortening velocity. In contrast, bladder preparations from homozygous knockout neonatal mice did not exhibit phase 1, but phase 2 could be observed, *i.e.* a similar isometric force and maximal shortening velocity, compared to wild-type phase 2. Thus, non-muscle myosin appears to be recruited in the sustained phase of smooth muscle contraction during prolonged KCl depolarization in the animal model used.

Upon stimulation with phorbol ester a similar sustained contraction was observed in both wild-type and knockout smooth muscle preparations. Therefore, non-muscle myosin may also be recruited during phorbol ester stimulation in both wild-type and knockout muscle preparations.

The participation of non-muscle myosin in smooth muscle contraction was further supported by the finding of longitudinally arranged intracellular filaments in cultivated smooth muscle cells from both wild-type and knockout mice by immunofluorescence microscopy, using a specific antibody raised against non-muscle myosin heavy chain. In

a similar way, smooth muscle myosin heavy chain structures were investigated in cultivated smooth muscle cells. As expected, longitudinally arranged intracellular filamentous structures of smooth muscle myosin were observed in wild-type smooth muscle cells, but not in smooth muscle cells from knockout mice.

In conclusion, in neonatal smooth muscle the initial phase of contraction elicited by KCl depolarization is generated by smooth muscle myosin heavy chain recruitment. Upon prolonged KCl depolarization non-muscle myosin is recruited in the sustained phase of contraction, as well as upon stimulation with phorbol ester. Thus, it was possible, for the first time, to verify the involvement of the non-muscle myosin in smooth muscle contraction *in vivo*. The results of the present study contribute to the understanding of the regulatory mechanisms of smooth muscle contraction.

ZUSAMMENFASSUNG

Das Ziel dieser Studie war es, die Beteiligung der nichtmuskulären schweren Myosinketten an der Kontraktion der glatten Muskeln unter physiologischen Bedingungen zu untersuchen. Als Versuchsmodell wurde die Harnblase von neugeborenen Wildtyp und transgenen Mäusen verwendet, bei denen das Gen für die glattmuskelspezifischen schweren Myosinketten durch „Gene Targeting“ funktionell eliminiert wurde (Knock-Out). Das Fehlen der Expression der glattmuskelspezifischen schweren Myosinketten wurde durch Elektrophorese und Immunfärbung bestätigt. Im Gegensatz dazu blieb die Expression der nichtmuskulären schweren Myosinketten unverändert.

Die mechanische Analyse des glatten Muskels wurde mit intakten Muskelpräparaten aus der Harnblase durchgeführt. Das Muskelpräparat wurde in KCl-Lösung oder mit Phorbol ester stimuliert. Die Aktivierung mittels depolarisierender KCl-Lösung führte bei neugeborenen Wildtyp Mäusen zuerst zu einer transienten Kontraktion (Phase 1) mit hoher Kraftentwicklung und maximaler Verkürzungsgeschwindigkeit, und danach zu einer tonischen Kontraktion (Phase 2) mit niedrigerer Kraftentwicklung und maximaler Verkürzungsgeschwindigkeit. Blasenpräparate neugeborener Knock-Out Mäuse dagegen zeigten keine Phase 1, sondern nur eine tonische Kontraktion, die mit Wildtyp Mäusen vergleichbar war. Daher scheint nichtmuskuläres Myosin an der tonischen Kontraktion des glatten Muskels beteiligt zu sein.

Durch Stimulierung mit Phorbol ester waren ähnliche tonische Muskelkontraktionen der Blasenpräparate sowohl bei Wildtyp als auch bei Knock-Out Mäusen zu beobachten. Vermutlich wird also das nichtmuskuläre Myosin durch Stimulierung mit Phorbol ester aktiviert.

Intrazelluläre Filamente wurden durch Immunfluoreszenz mit einem spezifischen Antikörper gegen nichtmuskuläre schwere Myosinketten in kultivierten primären glatten Muskelzellen untersucht. Dabei zeigten die Muskelzellen sowohl von Wildtyp als auch von Knock-Out Mäusen intrazelluläre dicke Myosinfilamente, was für die Beteiligung des nichtmuskulären Myosins an der glatten Muskelkontraktion spricht. Entsprechend wurden

intrazelluläre Filamente mit einem Antikörper gegen glattmuskelspezifische schwere Myosinketten in kultivierten primären glatten Muskelzellen untersucht. Wie erwartet, konnten nur in glatten Muskelzellen von Wildtyp Mäusen intrazelluläre Filamente nachgewiesen werden, nicht aber in denen von Knock-Out Mäusen.

In dieser Arbeit konnte zum ersten Mal gezeigt werden, dass nichtmuskuläres Myosin zumindest an der tonischen Kontraktion glatter Muskelzellen beteiligt sein kann.

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig und ohne unzulässige Hilfe Dritter angefertigt habe. Die benutzten Hilfsmittel sowie die Literatur sind vollständig angegeben.

Berlin, den 07. Dezember 2001

Valéria Lamounier Zepter