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The smooth muscle myosin seven amino acid heavy chain insert's kinetic role in the crossbridge cycle for mouse bladder

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The seven amino acid insert in the smooth muscle myosin heavy chain is thought to regulate the kinetics of contraction, contributing to the differences between fast and slow smooth muscle. The effects of this insert on force and stiffness were determined in bladder tissue of a transgenic mouse line expressing the insert SMB at one of three levels: an SMB wild type (+/+), an SMA homozygous type (−/−) and a heterozygous type (+/−). For skinned muscle, an increase in MgADP or inorganic phosphate (P_i) should shift the distribution of crossbridges in the actomyosin ATPase (AMATPase) to increase the relative population of the crossbridge state prior to ADP release and P_i release, respectively. Exogenous ADP increased force and stiffness in a manner consistent with increasing the Ca^{2+} concentration in both the +/+ and +/− mouse types. However, the −/− type showed a significantly greater increase in force than in stiffness suggesting that immediately prior to ADP release, the AMATPase either has an additional force producing isomerization state or a slower ADP dissociation rate for the −/− type compared to the +/+ or +/− types. Exogenous P_i led to a significantly greater decrease in stiffness than in force for all three mouse types suggesting that there is a force producing state prior to P_i release. In addition, the increase in P_i showed similar changes in the +/+ and −/− types whereas in the +/− type the decreases in both force and stiffness were greater than the other two mouse types indicating that the insert can affect the cooperativity between myosin heads. In conclusion, the seven amino acid insert modulates the kinetics and/or states of the AMATPase, which could lead to differences in the kinetics of contraction between fast and slow smooth muscle.

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Smooth muscle has been dichotomized as fast and slow depending on the kinetics of muscle contraction. The fast and slow contractile properties of skinned, thiophosphorylated smooth muscle tissue are preserved using photolytic release of MgATP implicating that differences in the actomyosin ATPase (AMATPase) crossbridge cycle contribute to the differences in the contractile properties of smooth muscle (Horiuti *et al.* 1989). Although actin is not conserved among all smooth muscle tissue, the different isoforms have been shown to have negligible effects on the AMATPase activity *in vitro* (Mossakowska *et al.* 1985), implicating myosin as the cause of the differences in the contractile properties. The smooth muscle myosin molecule is composed of two heavy chains (MHC), two essential light chains (MLC_{17}) and two regulatory light chains (MLC_{20}). Several isoforms of each are expressed in smooth muscles.

The MLC_{17} has an alternatively spliced exon producing two isoforms, MLC_{17a} and MLC_{17b}, differing by their isoelectric points (Hasegawa & Morita, 1992). Faster smooth muscle generally expresses a higher level of MLC_{17a} (Malmqvist & Arner, 1991), where changes in MLC_{17a}/MLC_{17b} between different smooth muscles correlate to differences in shortening velocity and ATPase rates (Helper *et al.* 1988; Malmqvist & Arner, 1991) but this finding is not universal (Meer & Eddinger, 1997; Sherwood & Eddinger, 2002). The MLC_{20} also expresses two isoforms separated by their acidity but only one isoform has been reported in postnatal tissue (Inoue *et al.* 1989). The MHC expresses two pairs of isoforms. At the tail region are SM1 and SM2 (Rovner *et al.* 1986; Nagai *et al.* 1989). Our interest is in the amino terminus MHC isoform where an alternatively spliced exon results in the presence (SMB) or absence (SMA) of seven amino acids near the nucleotide binding site (Hamada *et al.* 1990; White *et al.* 1993; Kelley *et al.* 1993). Since nucleotide attachment and detachment to the actomyosin complex plays an integral role in force development during the crossbridge cycle, changes in the myosin molecule near the nucleotide binding site may affect nucleotide affinity and thus change certain rate constants of the crossbridge cycle. Many studies have

shown a correlation between the expression level of SMA/SMB and the speed of the crossbridge cycle in smooth muscle, finding the presence of the insert resulted in a shorter duty cycle (Lauzon *et al.* 1998), a faster motility velocity (Rovner *et al.* 1997) and a faster shortening velocity (Eddinger & Meer, 2001). A general pattern has emerged showing faster muscles express higher levels of both SMB and MLC_{17a} making conclusions about the role of an individual isoform equivocal at best (Sjuve *et al.* 1996). Therefore any work that deals with intact tissue cannot isolate the cause to just one variable. On the other hand, experiments that are able to manipulate only the level of SMA/SMB have been performed using purified myosins and thus cannot mimic the environment of intact tissue.

We investigated whether changes in the SMA/SMB expression affects the crossbridge cycle in smooth muscle using a transgenic mouse line (Babu *et al.* 2001). This line is a knockout of SMB allowing for different expression levels of SMA/SMB in bladder without changing the expression levels of SM1/SM2 or MLC_{17a}/MLC_{17b} (Babu *et al.* 2001). This has allowed us to categorize three mouse types: predominantly expressing SMB (+/+); predominantly expressing SMA (−/−; SMB knockout); and a heterozygous expression (+/−).

Our work examines two steps in the crossbridge cycle and how changes in the expression level of SMA/SMB may influence crossbridge states. The crossbridge cycle describes the kinetics of muscle force generation through a series of states that involve the association and dissociation of actin (A) and myosin (M) along with MgATP and its hydrolysis products, ADP and P_i as shown in Scheme 1 and Fuglsang *et al.* (1993).

We probed two steps in the crossbridge cycle, the release of P_i and ADP. The addition of either exogenous inorganic P_i or MgADP shifts the equilibrium at their respective release points in the cycle, perturbing the crossbridge population by increasing the relative population of crossbridges in the state(s) immediately prior to either P_i or ADP release. Observing force and stiffness allows us to determine if the heavy chain insert adds or circumvents any of the states immediately preceding P_i or ADP release and also if the population of these state(s) varies due to the relative expression of SMA/SMB.

The kinetics of the bladder did differ depending on the expression level of the heavy chain NH_2 insert. The −/− type showed that immediately prior to ADP release from the actomyosin complex, either an additional step in the crossbridge cycle is present or the single state has a slower ADP off-rate than either the +/+ or +/− types. In all three types, there was evidence of a force generating state immediately prior to the release of P_i from the actomyosin complex, however the population of this state is significantly greater in the two homozygous animals compared to the heterozygous one.

METHODS

Animals

The method for cloning and generation of transgenic mice expressing the different SMA/SMB levels has been published in detail (Babu *et al.* 2001). Bladder tissue from adult mice was used for all mechanical experiments. Mice were killed by exposure to a rising concentration of CO_2 in accordance with the ethical treatment of animals using a protocol approved by the Case Western Reserve University Institutional Animal Care and Use Committee. Mice were categorized as homozygous positive (+/+; SMB homozygous), heterozygous (+/−) or homozygous negative (−/−; SMB knockout) for the heavy chain NH_2-terminal insert.

Histology

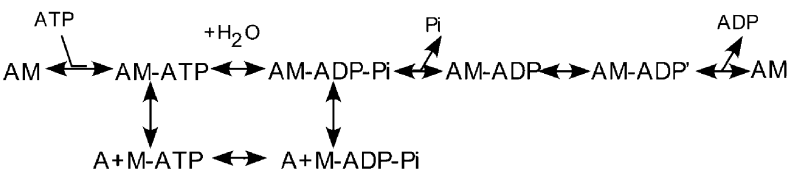
Bladder tissues from wild type and SMB −/− null mice were fixed in 10 % neutral buffered formalin, dehydrated through a gradient of alcohols, embedded in paraffin, sectioned, and stained with haematoxylin and eosin.

Quantitative immunoblot analysis

Quantitative immunoblotting was carried out as described previously (Babu *et al.* 2001). Briefly, total proteins isolated from bladder and aorta were separated on a 12 % SDS-polyacrylamide gel (PAGE) and transferred to nitrocellulose membrane. Membranes were probed with the following primary antibodies: mouse mouse monoclonal anti-smooth muscle actin, mouse monoclonal anti-myosin light chain 20 (both from Sigma), mouse monoclonal anti- MLC_{17} antibodies. The signals were detected by Super signal West Dura substrate and quantified by densitometry scanning and analysed using IMAGE software (Version 6.1, National Institutes of Health).

Force

The bladder was removed and placed into Ca^{2+}-free physiological saline solution (PSS) solution (mM): 140 NaCl, 4.7 KCl, 1.2 $NaH_2PO_4.7H_2O$, 2.0 MOPS, 0.02 EDTA, 1.2 $MgCl_2.6H_2O$, 5.6 glucose and 0.5 EGTA, pH 7.0). Bladder strips were cleared of connective tissue and then cut into strips approximately 400–1000 μm long, 300–500 μm wide and 150–300 μm thick. As previously described (Rhee & Brozovich, 2000), aluminum foil



Scheme 1.

Table 1. Solution constituents

pCa	KMS (mM)	EGTA (mM)	BES (mM)	$MgCl_2$ (mM)	$CaCl_2$ (mM)	ATP (mM)	CP (mM)
9.0	54.87	10	25	7.33	0.02	5.4	25
5.8	39.38	10	25	7.04	7.96	5.4	25
5.4	37.18	10	25	7.00	9.08	5.4	25
5.0	36.08	10	25	6.98	9.63	5.4	25
4.4	35.24	10	25	6.98	10.00	5.4	25
4.0	34.57	10	25	6.97	10.22	5.5	25
1% Triton	25	5	5	6.9	0	5	25
4.0 + 5mM MgADP	113.53	5.0	25	8.01	5.32	5.5	0
4.0 + 10 mM P_i	107.94	5.0	25	7.58	5.2	5.3	0

KMS, potassium methane sulfonate; BES *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid. Triton solution includes an additional 5 % (v/v) $CaCl_2$. All solutions include calmodulin 250 u ml^{-1} and 20 u ml^{-1} creatine phosphokinase except the ADP solution which had no creatine phosphokinase.

T-clips were attached to each end of the bladder strips. The tissue was then immersed in pCa9 solution where one end was hooked to a force transducer (Akers AE 801 MEMSCAP, San Jose, CA, USA) and the other to a piezoelectric length driver (Physik Instrumente, Waldbron, Germany) or a servomotor (Aurora Scientific, Aurora, ON, Canada) on a mechanics workstation. The tissue was stretched to a level sufficient to just develop tension and then by an additional 30 %, defining the length L_o where force is a maximum (data not shown). Bladder strips were skinned in 1 % Triton solution at pCa4 similar to that described by Sward *et al.* (1998). Tissue was left in the skinning solution until force reached a steady state, approximately 20–40 min. Tissue was then returned to pCa9 solution where force relaxed to the baseline. Strips were activated with Ca^{2+}. Upon reaching a steady state, the strip was transferred into 5 mM MgADP solution, 10 mM P_i solution or a different pCa level (5.8, 5.4, 5.0 or 4.0). The order of the pCa solutions varied between experiments.

Stiffness

After reaching a steady state force at any pCa level or in a nucleotide solution, the length driver oscillated with a peak–peak sine wave at an amplitude of 0.3 % L_o and at a frequency of 40 and 80 Hz. The length of the motor displacement and the corresponding tension of the tissue were individually transformed into a Fourier series (Shue & Brozovich, 1999). Stiffness was defined as the magnitude of the force transform divided by the length transform. Stiffness was normalized by cross-sectional area, such that the stiffness in pCa4 was defined as 100 % and in pCa9 was defined to be 0 %. Stiffness was the same at both 40 and 80 Hz, therefore only the data at 40 Hz is reported in the text.

Solutions

All solutions were prepared using a computer program that calculated the amount of stock solutions required for a set of free ion concentrations adjusting for temperature, pH and ionic strength (Brozovich & Yamakawa, 1995). Temperature was set at 25 °C. Solutions were prepared with a pH of 7.0. Ionic strength was set at 200 mM. Specific concentrations for each solution are provided in Table 1.

Statistics

The Student's paired *t* test was used to compare the differences for the changes of force and stiffness with a level of significance $P < 0.05$. Data in the text are given as means ± S.E.M.

RESULTS

Tissue histology shows no significant differences between SMB wild type mice bladder strips and SMB knockout mice bladder strips (Fig. 1). The smooth muscle orientation was similar between the +/+ wild type mice and the −/− double knockout mice. Since the force measured is dependent on the cosine of the angle of the muscle fibres and the force transducer, only orientations with very large change in angles would affect the total forces measured. Therefore, orientation would not contribute to any significant differences in force.

Changes in the SMB expression level did not affect the expression level of other myosin isoforms including MLC_{17} or MLC_{20} (Fig. 2). Only one band was seen for either the MLC_{17} or MLC_{20} in all three mouse types indicating no differences between the mice.

Overall forces in pCa4 are given in Table 2. Our results indicate that the insert has no role in total force generation, contradicting the findings from Babu *et al.* (2001). A possible explanation is the method of activation. Whereas we activated permeabilized tissue to high levels of Ca^{2+}, the others used KCl activation on intact membranes. Also, laser trap studies suggest the insert does not affect unitary force or total average force (Lauzon *et al.* 1998), in accordance with our results.

Before studies comparing the effects of MgADP and P_i on the crossbridge kinetics of each mouse type, we first established a force–stiffness relationship at different Ca^{2+} levels to determine if any effect we observed was equivalent to changing the number of activated crossbridges. As the muscle was activated with Ca^{2+}, both the steady state force and stiffness increased. The relationship between force and stiffness over various pCa levels (Fig. 3) showed the following linear relationship:

$$\text{Force} = (0.94 \times \text{stiffness}) - 0.07.$$

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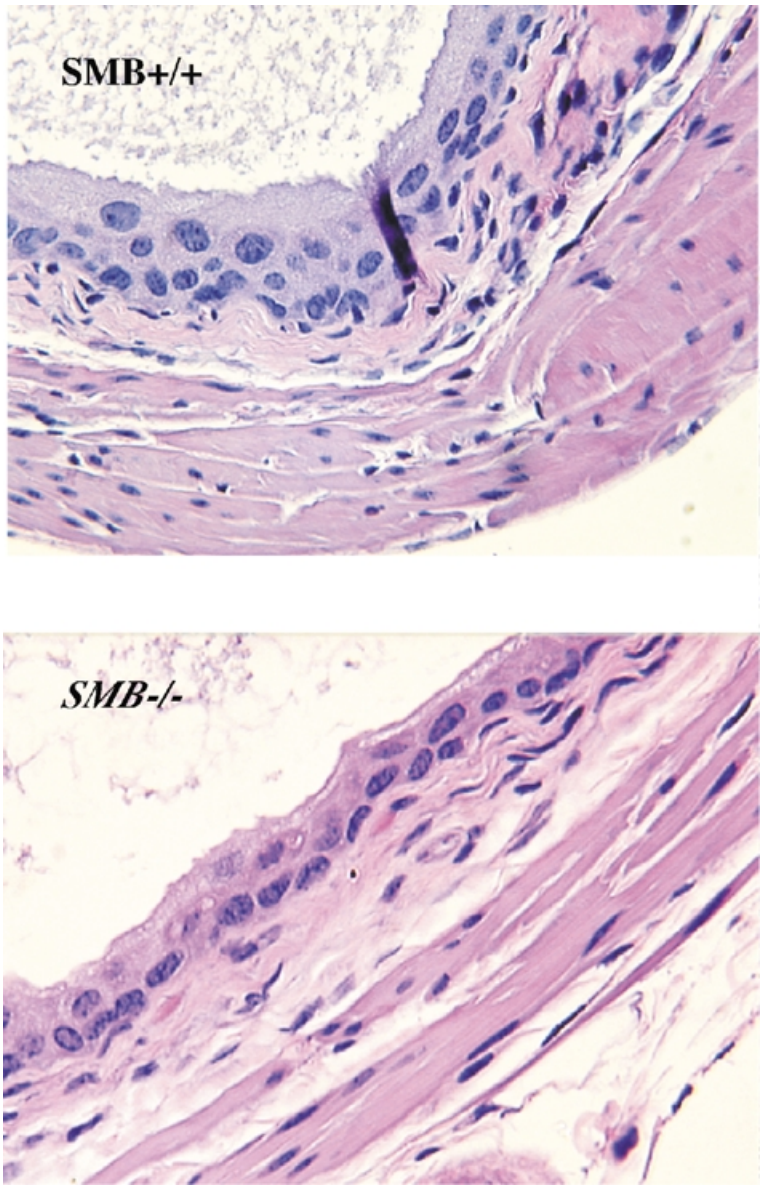


Figure 1. Histological staining of wild-type and SMB −/− bladder

Sections were stained with haematoxylin and eosin, × 40 magnification.

To investigate the state of the crossbridge cycle immediately before ADP release from the myosin head, the skinned bladder was transferred from pCa4 to pCa4 with 5 mM MgADP, which resulted in an increase in both force and stiffness for all three mouse types (Fig. 4*A*). For 5 mM MgADP in pCa4 *vs.* pCa4 alone, the +/+ type resulted in an increase in force of 67 % while the stiffness increased by 63 %, the +/− type had a force increase of 52 % and a stiffness increase of 39 % while the −/− type had a 65 % force increase and a 36 % stiffness increase (Table 2).

In pCa4 with 5 mM MgADP *vs.* pCa4 alone, the changes in force and stiffness for each mouse type were compared to determine if results deviated from the force-stiffness

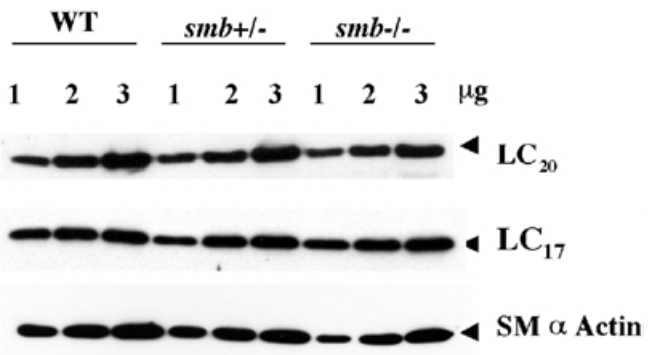


Figure 2. Western blot analysis of light chain isoform expression

Three different concentrations of total proteins extracted from SMB+/+ (WT, wild-type), SMB+/−, and SMB−/− mouse bladder were resolved on a 12 % SDS-PAGE and immunoblotted with LC20, smooth muscle actin or LC17-specific antibody.

Table 2. Force and stiffness

	+/+	+/−	−/−
Absolute values in pCa4			
Force (mN mm^{-2})	7.63 ± 1.54	7.47 ± 0.96	7.60 ± 0.96
Stiffness at 40 Hz (mN mm^{-1})	42.35 ± 7.71	36.23 ± 5.45	34.45 ± 4.48
No. of muscle strips	16	17	25
No. of animals	5	5	9
Relative values in ADP			
Force	167 ± 15 %	152 ± 8 %	165 ± 10 %
Stiffness	163 ± 23 %	139 ± 12 %	136 ± 6 %
No. of muscle strips	9	7	14
No. of animals	3	2	5
Relative values in P_i			
Force	67 ± 5 %	44 ± 5 %	62 ± 5 %
Stiffness	28 ± 8 %	11 ± 8 %	24 ± 6 %
No. of muscle strips	7	10	11
No. of animals	2	3	4

The +/+ condition represents SMB/SMB, −/− is the SMB knockout or the complete absence of the seven amino acid NH_2 terminal MHC insert (Kelley *et al.* 1993) and +/− is the heterozygous state. Force and stiffness at pCa4 are defined as 100 %. Percentages listed for ADP and P_i are relative to pCa4 values. The changes in force and stiffness for the +/− type in the P_i condition were statistically significant compared to the other two mouse types ($P < 0.05$). All values are given as means ± S.E.M.

relationship when the muscle was activated with only Ca^{2+} (Fig. 3). Only the −/− type showed a crossbridge behaviour that deviated from the Ca^{2+} force–stiffness relationship where the increase in force was significantly greater than the increase in stiffness, $P < 0.05$. On the other hand, for both the +/+ and +/− mouse types, the increases in force and stiffness were not statistically different.

To investigate the state of the crossbridge cycle immediately before P_i release from the myosin head, the skinned bladder was transferred from pCa4 to pCa4 with 10 mM P_i; both steady state force and stiffness decreased in all three mouse types (Fig. 4*B*). For 10 mM inorganic P_i in pCa4 *vs.* pCa4 alone, the +/+ type had force decreased by 33 % while stiffness decreased by 72 %, the +/− type had a force

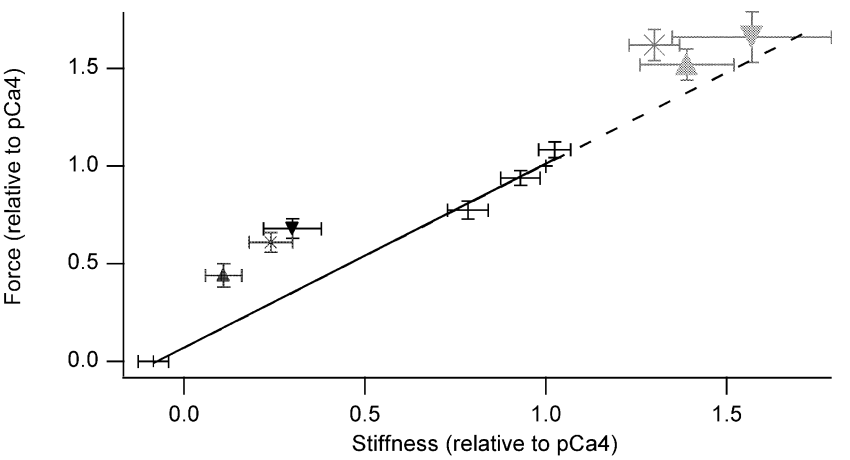
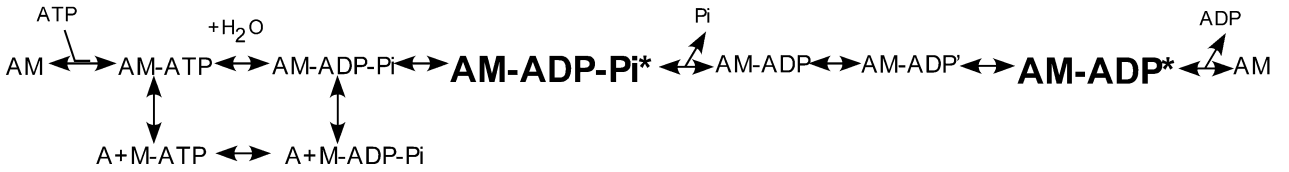


Figure 3. The force–stiffness relationship

Five different force and corresponding stiffness values were observed at pCa values of 5.8, 5.4, 5.0, 4.4 and 4.0 ($n = 10$) and fit to a line, $r^2 = 0.99$ (black line, +). Force *vs.* stiffness changes: ▼ = +/+, ▲ = +/−, × = −/−; P_i = black, ADP = grey. Force and stiffness in pCa4 were defined as 100 %. The relationship between steady state force and steady state stiffness during Ca^{2+} activation is linear. In the ADP condition, only the −/− mouse type significantly deviated from the force *vs.* stiffness regression line at various pCa concentrations ($P < 0.05$). All three mouse types significantly deviated from the line in the P_i condition ($P < 0.05$).



Scheme 2.Changes to crossbridge cycle (see text for details)

decrease of 56 % and a stiffness decrease of 89 % while the −/− type had a 38 % force decrease and a 76 % stiffness decrease (Table 2). Unlike for MgADP, comparing pCa4 with 10 mM P_i *vs.* pCa4 showed all three mouse types had a significantly greater decrease in stiffness than in force ($P < 0.05$) and thus all three deviated from the Ca^{2+} force–stiffness relationship (Fig. 3).

DISCUSSION

Evidence indicates that varying the expression level of the myosin heavy chain seven amino acid insert (SMA/SMB) may play a significant role in the kinetics of smooth muscle contraction. Unfortunately, studies of smooth muscle strips are convoluted by several variations found between a fast smooth muscle and a slow smooth muscle that lie well beyond the expression level of SMA/SMB, including differences in expression levels of other myosin isoforms (MLC_{17}, and SM1/SM2). The transgenic mice in our study are unique in that their bladder tissue expresses the SMA/SMB at three different levels without changes in the expression of SM1/SM2, MLC_{17a}/MLC_{17b} or MLC_{20}. Thus in the bladder of this line of transgenic mice, any differences in the AMATPase cannot be attributable to changes in the expression or isoform ratios of SM1/SM2, MLC_{20} and MLC_{17} (Babu *et al.* 2001). This makes the bladder of these mice a preferable model to evaluate the effects of changes in the level of expression of SMA/SMB on the crossbridge cycle.

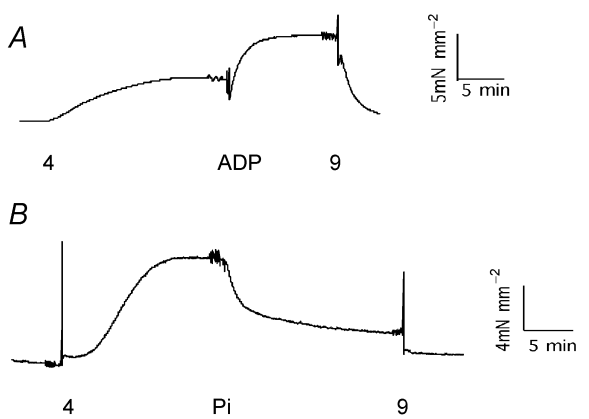


Figure 4. Force traces during Ca^{2+} activation

A, force trace during Ca^{2+} activation in 5 mM MgADP. Tissue was allowed to relax in pCa9 (9) before being shifted to pCa4 (4), pCa4 with 5 mM MgADP (ADP) and then back into pCa9. Ca^{2+} activation results in an increase in steady state force, which is further increased with MgADP. *B*, force trace during Ca^{2+} activation in 10 mM inorganic P_i. Tissue was allowed to relax in pCa9 before being shifted to pCa4 (4), pCa4 with 10 mM P_i (P_i), and then back into pCa9. Ca^{2+} activation results in an increase in steady state force, which is decreased by P_i.

The crossbridge cycle describes the development of force through a series of complexes between actin (A), myosin (M), MgATP and its hydrolysis products, ADP and P_i (Scheme 1). Beginning in the rigor state (AM), ATP binding to AM results in rapid dissociation forming an A + M-ATP state followed by hydrolysis of ATP by myosin. P_i release from the A + M-ADP-P_i state is thought to result in crossbridge attachment, AM-ADP, beginning the duty cycle. The AM-ADP state then isomerizes to a high force generating state (AM-ADP′) followed by ADP release returning to the rigor state (AM). MgATP subsquently binds to the AM state causing detachment, ending the duty cycle and recommencing another crossbridge cycle. While the crossbridge cycle for all muscle is frequently described in a generic manner, differences do exist between the kinetics of skeletal, cardiac and smooth muscle and even within different smooth muscle tissues, requiring changes in the crossbridge cycle to explain the differences in AMATPase rates (Rosenfeld *et al.* 2000).

The points of ADP release and P_i release in the crossbridge cycle can be studied with the addition of excess ADP or inorganic P_i which results in shifting the equilibrium of the crossbridge cycle just before the ADP or P_i release and increasing the relative population of these crossbridge states. By measuring force and stiffness, one can observe the properties of the crossbridge immediately before the release of ADP or P_i to identify if the crossbridges are attached or detached, and should they be attached whether the state is a force generating state or a non-force generating state.

Elevation of exogenous MgADP is thought to promote a bound, force generating state (AM-ADP′ in Scheme 1). This was found in all three mouse types. Placing the tissue in 5 mM MgADP resulted in an increase in force and stiffness, however only the −/− form deviated from the Ca^{2+} force–stiffness relationship where force increased by 65 % but stiffness by only 36 %. The −/− results imply a change in the properties of the crossbridge state. The attached crossbridges exerted a greater force than had they been activated by simply increasing the Ca^{2+} concentration. In contrast, by not deviating from the Ca^{2+} force–stiffness

relationship, the addition of exogenous MgADP for both the +/+ and +/− mouse types was equivalent to increasing the number of activated crossbridges attached to actin (67 % force and 63 % stiffness for +/+, 52 % force and 39 % stiffness for +/−). For the −/− type, significantly increasing the force per stiffness suggests either an additional step in the crossbridge cycle where the AM-ADP′ state isomerizes to produce a greater force (AM-ADP*), an isomerization not seen in either the +/+ or the +/− condition (Scheme 2) or simply a longer lived AM-ADP′ state due to a slower ADP off-rate in the −/− type resulting in a longer duration of a force generating state.

In skeletal muscle it has been suggested force can increase by having myosin heads bind in a stereospecific manner without increasing the number of crossbridges bound to the actin filament (Bershitsky *et al.* 1997). The absence of the NH_2 terminal insert (−/−) may induce similar behaviour in smooth muscle. According to Scheme 1, crossbridges are already bound just prior to ADP release and realign to produce a force, but the absence of the insert could result in a stereospecific readjustment of these crossbridges immediately before ADP release to slow ADP release or cause crossbridges to exert more force without increasing the number of crossbridges attached. For the +/+ and +/− mouse types, force increases proportionally to the number of crossbridges, suggesting this reorientation of crossbridges does not occur when at least one head expresses the insert. Should the crossbridges exert more force at certain stages of the crossbridge cycle, the overall force per cross-sectional area should be highest in the −/− type. Forces, however, did not show a statistically significant difference between the three mouse types supporting the theory that the actomyosin interaction has the same unitary force independent of the heavy chain expression (Lauzon *et al.* 1998). There may in fact be a difference, but that it is not detectable at 5 mM ATP. Lauzon *et al.* (1998) showed that while the relative duty cycle between having the insert present and absent remains constant at approximately a twofold difference independent of ATP concentrations, the absolute values decrease significantly with increasing ATP. Our experiments were done using 5 mM ATP, five times greater than the maximal ATP concentration used in Lauzon *et al.*'s work. Therefore, while the difference may be significant, it may be so slight that it can only be seen if the bound state is stabilized with high ADP concentration.

Past studies have investigated why smooth muscle has a longer duty cycle than skeletal muscle, to try and explain smooth muscle's greater efficiency. Rosenfeld *et al.* (2000) suggested that an additional AM-ADP state exists in the smooth muscle AMATPase compared to the AMATPase in skeletal muscle, which would contribute to a longer duty cycle in smooth muscle. In addition, the extra rotation in the neck region at the end of the smooth muscle power stroke compared to skeletal muscle (Whittaker *et al.* 1995; Gollub *et al.* 1996) may also contribute to a longer duty cycle. Analogously, an extra AM-ADP state (Scheme 2) or a slower rate of ADP dissociation may be due to the absence of the heavy chain insert, which could result in an additional rotation of the myosin molecule while bound to the actin filament. If, however, it is not an additional state but rather the two S1 heads of a single myosin molecule realigning along the actin filament, additional time may be needed to achieve this realignment, slowing the ADP off-rate and resulting in a longer duty cycle.

Without knowledge of all the rate constants in the crossbridge cycle for fast and slow smooth muscle, one can only loosely connect our conclusions regarding ADP release with other studies. Khromov *et al.* (1995) have shown that fast smooth muscle has a faster off-rate for ADP than slow smooth muscle. However, the faster rate at this step may be compensated for in other steps in the crossbridge cycle. Laser trap work has shown a shorter duty cycle for myosin expressing the insert due to a change in the ADP off-rate (Lauzon *et al.* 1998). Assuming rates are approximately equal for the common steps in the cycle, an additional step similar to an additional isomerization (AM-ADP′ $\rightleftharpoons$ AM-ADP*, Scheme 2) would result in a longer time to process through the states while actin and myosin are interacting. Alternatively, a slower transition between AM-ADP′ $\rightleftharpoons$ AM could also produce a longer duty cycle, and thus, an overall slower crossbridge cycle in the −/− type.

The NH_2 insert is located in loop 1 near the ADP binding site of myosin. Studies have shown a positive relationship between a longer loop and a quicker release of ADP, although the physiological significance remains in question (Sweeney *et al.* 1998). These results would suggest that the absence of the seven amino acid insert (SMA) causes a slower release of ADP and a longer duty cycle. The observations made with the −/− bladder tissue support this conclusion; the notion a longer loop 1 releases ADP faster is consistent with our findings since the −/− type, having a shorter loop length, would have a longer duty cycle be it from an additional AMADP isomerization (Scheme 2) or a longer time constant in the AM-ADP′ state. However since neither the +/− nor +/+ types deviate from the Ca^{2+} force–stiffness line, it can be concluded that only one head needs to have a longer loop 1 in order to reduce the duty cycle.

It is thought that upon P_i release, actin and myosin bind to generate force (Scheme 1). This is seen by the fall in force and stiffness in pCa4 with 10 mM inorganic P_i compared to pCa4 alone. Additionally, because the drop in stiffness was greater than the drop in force, we can surmise that immediately prior to P_i release there exists a force generating state. This is seen in Fig. 3 where those crossbridges that are bound generate a force in a manner that deviates from the Ca^{2+} force–stiffness relationship. Force development before P_i release necessitates crossbridges to be bound to

actin filaments, significantly modifying the model for the smooth muscle AMATPase. We propose that there exist two states between actin and myosin detachment and the P_i release, both involving crossbridge attachment. Following the A + M-ADP-P_i step, there is a low force producing step, AM-ADP-P_i, followed by an isomerization to a higher force state, AM-ADP-P_i* (Scheme 2). Skeletal and cardiac muscle studies have shown a similar transition from a non-force generating AM-ADP-P_i complex to a force generating AM-ADP-P_i* state prior to P_i release (Iwamoto, 1995; Dantzig *et al.* 1992; Ranatunga, 1999; Wang & Kawai, 1997). However, empirically this has not been seen. If more crossbridges were in a force generating state then one would expect to see an increase in the force per stiffness ratio as we see in smooth muscle. It may be that because smooth muscle is slower than striated muscle, we did see this effect where others could only deduce the existence of the higher force generating state. The data show the two homozygous conditions (+/+, −/−) have approximately equal populations of this state, and that it is higher than the population found in the heterozygous type (+/−). The falls in force and stiffness for the two homozygous cases were 38 % and 76 % for the +/+ and 33 % and 72 % for the −/− type, while the +/− type fell 56 % and 89 % for force and stiffness, respectively. That the fall in stiffness is greater than the fall in force when the tissue is immersed in high P_i solution indicates that the elevation of exogenous phosphate somehow favours the detachment of cross-bridges in the low force generating state.

While all three mouse types show evidence for this state, the differences in populations suggest some sort of cooperative behaviour between myosin heads in order to bind to actin prior to P_i release in the crossbridge cycle. The cause of this cooperativity is unclear. As hypothesized for the AM states prior to MgADP release, crossbridges may reorient themselves in a stereospecific manner immediately before P_i release, but the number of cross-bridges able to do this are far fewer than those detached from the actin filament since both force and stiffness decreased. The heterozygous condition (+/−) may not be as efficient in achieving this stereospecific orientation as the two homozygous conditions (+/+, −/−). Should the stereospecific orientation be more advantageous in homozygous mouse types, there should then exist a difference in the depression of force by P_i between different smooth muscle types. Löfgren *et al.* (2001) reported that the sensitivity of force to P_i was greater for tania coli than aorta. While their work dealt with the guinea-pig, it has been shown tania coli expresses 15–20 % SMB in mice (Siegman *et al.* 1997) while SMB is not expressed in chicken aorta (Fisher *et al.* 1997). It is possible that the asymmetrical heads are somehow disadvantaged to bind to actin in a P_i-bound state, but this describes not a kinetic role for the insert but rather a mechanical one, which is plausible and still describes a pertinent role for the insert.

Other factors beyond the SMA/SMB expression level could also be involved in the differences in the contractile properties between various smooth muscle tissues. SM1 and SM2 describe two heavy chain isoforms found at the carboxyl end of the myosin molecule (Rovner *et al.* 1986; Nagai *et al.* 1989). Data remain inconclusive regarding their roles. Some investigators have found the expression level of SM1/SM2 does affect the shortening velocity (Hewett *et al.* 1993). However, other investigators have not seen changes in shortening velocity (Meer & Eddinger, 1997; Sherwood & Eddinger, 2002) or in the motility assay (Kelley *et al.* 1992) and have attributed the carboxyl isoforms not to kinetics, but rather to filament assembly (Rovner *et al.* 2002). Similarly, the MLC_{17} has two isoforms thought to contribute to smooth muscle kinetics, MLC_{17a} and MLC_{17b} (Malmqvist & Arner, 1991; Matthew *et al.* 1998). Others, however, consider their role secondary having shown no correlation between the MLC_{17a}/MLC_{17b} expression levels and shortening velocity in single smooth muscle cells (Eddinger *et al.* 2000).

While most attention to smooth muscle kinetics is devoted to the activation of the actomyosin complex, differences in the dephosphorylation properties of the MLC_{20} between fast and slow muscle may also be responsible. It has been shown that different smooth muscle responds differently to phosphatase inhibitors influencing the rate of relaxation in a manner independent of the crossbridge cycle (Gong *et al.* 1992). It is not possible to rule this out as an explanation. However, that does not dismiss the role of the heavy chain. It is thought the heavy chain does interact with the myosin light chain phosphatase although not necessarily near loop 1 (Hartshorne, 1998). Nevertheless, isoforms in the heavy chain may affect this interaction and so would still have a kinetic influence on smooth muscle.

The bladder of this transgenic mouse line had an upregulation of calponin in both the hetereozygous mice (+/−) and the homozygous mice (−/−; SMB knockout). On the other hand, caldesmon was downregulated in the two (G. Babu and M. Periasamy, unpublished observations). Both caldesmon (Katsuyama *et al.* 1992; Malmqvist *et al.* 1996) and calponin (Winder & Walsh, 1993; Horowitz *et al.* 1996) have been suggested to modulate smooth muscle activation; however the role of the thin filament in smooth muscle activation is controversial (Adam *et al.* 1995; Brozovich & Yamakawa, 1995; Matthew *et al.* 2000). Calponin has been suggested to influence maximal muscle shortening velocity (Jaworoski *et al.* 1995; Matthew *et al.* 2000), but this is not a constant finding (Facemire *et al.* 2000). Furthermore, calponin has been reported to decrease on isometric force (Uyama *et al.* 1996) but others have not seen this effect (Jaworoski *et al.* 1995; Matthew *et al.* 2000). Differences in caldesmon content have been reported to affect force or stiffness of isometric contractions at submaximal Ca^{2+} activation but not at maximal levels

(Szpacenko *et al.* 1985; Malmqvist *et al.* 1996) as employed in this study. These two studies were done by either adding exogenous caldesmon or by extracting it from the tissue. In contrast, one group even showed a decrease in caldesmon resulted in a decrease in isometric force using KCl activation (Earley *et al.* 1998). Whereas studies observing the insert have only shown faster kinetics in the presence of the insert, the work regarding calponin and caldesmon has not shown a consistent pattern regarding their effects on smooth muscle contractility. Consequently, while we cannot completely dismiss the influence of calponin or caldesmon on our results, we think their contribution, if any, is secondary to that of the insert.

Other contributors to variations in the cycle may be tissue architecture. The tissue itself produces an internal load slowing the shortening velocity (Harris & Warshaw, 1990; Ogut & Brozovich, 2000). Purified proteins or proteins expressed using a baculovirus system avoid problems of internal loads and tissue architecture; however, the folding of myosin, actin and any other relevant proteins under the aforementioned conditions is not necessarily comparable to the folding of these proteins in their endogenous environments (Li *et al.* 2000). The different folding properties may contribute to the effects seen when manipulating the expression level of SMA/SMB, effects that do not actually exist when the proteins are expressed naturally in their respective tissues. Because the bladder tissue of this mouse line is able to express different expression ratios of SMA/SMB without changing the expression levels of any other major myosin isoforms or proteins, differences found in the contractile kinetics of the bladder can be attributed to the SMA/SMB expression level more confidently than in other experiments.

In conclusion, exogenous ADP demonstrated either an additional state in the crossbridge cycle for the −/− type (SMB knockout) not found for either the +/+ or +/− cases or a longer ADP binding time. Either condition may contribute to a longer duty cycle consistently found for the −/− form in *in vitro* studies leading to a longer overall crossbridge cycle due to bound crossbridges reorienting themselves in a manner that increases the efficacy of force production.

Exogenous P_i studies showed that all three mouse types have two states between detached crossbridges and P_i release, the first being a low force producing state, the second a high force producing state. Once in the high force producing state, crossbridges continue on through the crossbridge cycle unlike the low force producing ones which can return to the detached state in high levels of P_i, explaining why stiffness dropped more than force. While all three mouse types showed evidence for these two additional states, the two homozygous cases had a higher population of the high force generating state than the heterozygous type. While this does not suggest any differences at this point in the crossbridge cycle between the three mouse types, it does imply a cooperative relationship between the two myosin heads suggesting a mechanical role for the heavy chain insert.

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