Effects of h1-calponin ablation on the contractile properties of bladder *versus* vascular smooth muscle in mice lacking SM-B myosin

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The functional significance of smooth muscle-specific h1-calponin up-regulation in the smooth muscle contractility of SM-B null mice was studied by generating double knockout mice lacking both h1-calponin and SM-B myosin. The double knockout mice appear healthy, reproduce well and do not show any smooth muscle pathology. Loss of h1-calponin in the SM-B null mice bladder resulted in increased maximal shortening velocity (V_{max}) and steady-state force generation. The force dilatation pressure, which was decreased in the SM-B null mesenteric vessels, was restored to wild-type levels in the double knockout vessels. In contrast, the half-time to maximal constriction was significantly increased in the double knockout vessels similar to that of SM-B null mice and indicating decreased shortening velocity in the double knockout vessels. Biochemical analyses showed that there is a significant reduction in smooth muscle α-actin levels, whereas h-caldesmon levels are increased in the double knockout bladder and mesenteric vessels, suggesting that these changes may also partly contribute to the altered contractile function. Taken together, our studies suggest that up-regulation of h1-calponin in the SM-B null mice may be necessary to maintain a reduced level of cross-bridge cycling over time in the absence of SM-B myosin and play an important role in regulating the smooth muscle contraction.

(Resubmitted 7 August 2006; accepted after revision 7 September 2006; first published online 14 September 2006)
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In smooth muscle, the kinetics of the actin–myosin interaction are considered to be the major determinant of the maximal shortening velocity (V_{max}) and rate of tension development (reviewed in Somlyo *et al.* 2004). The contractile properties of various smooth muscle tissues are well correlated to different smooth muscle myosin heavy and light chain isoforms (Matthew *et al.* 1998; Somlyo *et al.* 1998; Lofgren *et al.* 2002; Arner *et al.* 2003). Several *in vitro* studies suggest that the V_{max} is determined by the presence (SM-B myosin) or absence (SM-A myosin) of a 7 amino acid insert in the myosin head (reviewed in Babu *et al.* 2000; Karagiannis & Brozovich 2003). Using an *in vitro* motility assay, Kelley *et al.* (1993) have shown that the actin-activated ATPase activity and velocity of actin movement of myosin isolated from phasic smooth muscle is greater than that of tonic smooth muscle tissues. The femoral/saphenous arteries, which express predominantly the SM-B isoform, have a twofold higher ATPase activity and V_{max}, similar to phasic smooth muscle (DiSanto *et al.* 1997). Rovner *et al.* (1997) have expressed smooth muscle heavy meromyosin molecules with or without the 7 amino acid insert specific for SM-B myosin, and demonstrated that the SM-B myosin isoform had a twofold higher actin-activated myosin ATPase activity and actin filament velocity. Using laser trap experiments, Lauzon *et al.* (1998*a*,*b*) have shown at the level of a single myosin molecule that the 7 amino acid insert (specific for SM-B myosin) does not affect the myosin's ability to generate force and motion, but rather affects the rates at which the myosin transitions through the cross-bridge cycle. Specifically, the 7 amino acid insert increases both the rate of MgATP binding and MgADP release from the active site.

To determine the role of SM-B myosin in smooth muscle physiology, we have generated a mouse model

 DOI: 10.1113/jphysiol.2006.118828

deficient only in SM-B myosin (*smb*−/−). Removal of SM-B-specific exon 5B results in a switch to SM-A, but does not affect survival or cause any overt smooth muscle pathology (Babu *et al.* 2001). Physiological analysis reveals that loss of SM-B myosin results in a significant decrease in velocity of muscle shortening in bladder and in mesenteric vessels which express high levels of SM-B myosin (Babu *et al.* 2001, 2004; Karagiannis *et al.* 2003, 2004). These studies suggest that the 7 amino acid insert could be involved in the kinetics of smooth muscle contraction. However, there are reports which show no correlation between SM-B myosin content and mechanical performance of the smooth muscle (Haase & Morano, 1996; Siegman *et al.* 1997). In pregnant hypertrophied rat myometrium (Haase & Morano, 1996) and in the mouse megacolon of Hirschsprung's disease (Siegman *et al.* 1997) the maximal shortening velocity was increased, while the SM-B myosin isoform content was decreased. We have also observed altered expression of thin-filament proteins like h1-calponin (h1-CaP) and h-caldesmon (h-CaD) in the *smb*−/− mice, suggesting that these proteins could possibly be involved in the regulation of smooth muscle contractility in *smb*−/− mice (Babu *et al.* 2004).

Calponin, an actin-binding regulatory protein, has been found in smooth muscle and non-muscle tissues and is encoded by three different homologous genes (reviewed by Morgan & Gangopadhyay, 2001). Basic (h1) CaP is the major calponin found in the smooth muscle and has been extensively studied (reviewed by Takahashi & Yamamura, 2003). Several *in vitro* studies have suggested that h1-CaP inhibits actin-activated myosin ATPase activity (Horiuchi & Chacko, 1991; Haeberle, 1994; Winder *et al.* 1998) in a dose-dependent manner (Itoh *et al.* 1994; Jaworowski *et al.* 1995; Uyama *et al.* 1996). Ablation of h1-CaP resulted in faster unloaded shortening velocity of smooth muscle contraction (Matthew *et al.* 2000; Takahashi *et al.* 2000; Fujishige *et al.* 2002). The finding that h1-CaP levels are increased in the SM-B null mice (Babu *et al.* 2004) may suggest that h1-CaP plays a role in the decreased velocity of shortening observed in this model. However, it is unclear whether the up-regulation of h1-CaP in the SM-B null mice is a compensatory mechanism to maintain function or the cause for a decrease in contractile function. To critically assess the role of h1-CaP in SM-B null mice, we generated a double knockout mouse model lacking both h1-CaP and SM-B and studied smooth muscle contractility of non-vascular (urinary bladder) and vascular (mesenteric arteries) tissues.

Methods

Animals were killed by exposure to rising concentrations of CO_2, in accordance with the ethical treatment of animals, using a protocol approved by the Ohio State University and Case Western Reserve University, or by cervical dislocation and thoracotomy following isoflurane anaesthesia, according to a similarly approved protocol at the University of Vermont. The mice used for the following studies were F3 generations of both sexes and between 18 and 25 weeks old.

Generation of double knockout (*smb*−/− & *Cnn1*−/−) mice

The generation of SM-B myosin-isoform-specific knockout mice (Babu *et al.* 2001) and CaP null (*Cnn1*−/−) mice (Yoshikawa *et al.* 1998) has been described previously. In order to eliminate genetic differences among mouse strains, we first transferred mutated SM-B and CaP alleles to Black/Swiss background by backcrossing to Black/Swiss wild-type mice. Heterozygous mice from each generation (up to 6 generations) were crossed to wild-type Black/Swiss mice. After six crossings, the individual heterozygous mice were bred together to generate SM-B or CaP single knockout mice. To generate double knockout (*smb*−/− & *Cnn1*−/−) mice, SM-B and CaP null mice were bred together. The *smb*−/− & *Cnn1*+/− (heterozygous) mice were then crossed to generate *smb*−/− and double knockout (dKO) mice. Genotyping was carried out using the PCR method as described previously (Yoshikawa *et al.* 1998; Babu *et al.* 2001).

Reverse-transcription PCR analysis

To determine the expression levels of neutral (h2) and acidic (h3) CaP mRNA, reverse transcription (RT)-PCR analysis was carried out. Total RNA from wild-type (WT), *smb*−/− and dKO bladder tissues was extracted as described earlier (Babu *et al.* 2001). The reverse transcription of 2 μg of total RNA was carried out according to the manufacturer's manual using a StrataScript First-Strand Synthesis System (Stratagene, Catalogue no. 200420). Following oligo-(dT)-primed first-strand cDNA synthesis, a 1 μl portion of the first-strand cDNA mixture was subjected to PCR as described earlier (Yoshikawa *et al.* 1998) using the following primers: h2-CaP (forward 5′-CCCTCTGCCGGTCCCGCTGG-3′ and reverse 5′-CAAACTGATGTGAAGAGAT-3′), h3-CaP (forward 5′-AACAGCCAGACCCACTTCAAC-3′ and reverse 5′-TCGGGGTATTCTGCTGATAATC-3′ and mouse GAPDH (forward 5′-CCCATCACCATCTTCCAGGA-3′ and reverse 5′-TTGTCATACCAGGAAATGAGC-3′). The PCR products were analysed on 1.2% agarose gel electrophoresis and signals were quantified by UVP BioImaging systems. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used along with CaP primers as internal controls.

Histology

Bladder tissues from *smb*−/− and dKO mice were fixed in 10% neutral-buffered formalin, dehydrated through a gradient of alcohol, and embedded in paraffin. Sections were stained with haematoxylin and eosin as described previously (Karagiannis *et al.* 2003).

Western blot analysis

Western blotting was carried out as previously described (Babu *et al.* 2001, 2004). Briefly, total proteins extracted from bladder or mesenteric vessels were separated on 8% (for smooth muscle myosin isoforms SM1 and SM2), 10% (for smooth muscle α-actin, β-actin, γ-actin, h1-CaP and h-CaD) or 15% (for light chain 17 and light chain 20 (LC_{17} and LC_{20})) SDS-polyacrylamide gels (PAGE) and transferred to a nitrocellulose membrane. Membranes were probed with the following primary antibodies: sheep polyclonal anti-γ-actin (Chemicon), rabbit polyclonal antibodies specific for SM1, SM2, h1-CaP and h-CaD (Babu *et al.* 2004), mouse monoclonal antibodies specific for smooth muscle α-actin, β-actin, myosin light chain 20 (Sigma), and myosin light chain 17 as described previously (Babu *et al.* 2001, 2004). Signals were detected by SuperSignal West Dura extended substrate (Pierce) and quantified by densitometry.

Mechanical experiments

Bladder. All solutions and the methods for tissue preparation are described in detail elsewhere (Karagiannis *et al.* 2003), and solutions included 250 units ml^{-1} of calmodulin. Briefly, bladder tissues were cut into strips of approximately 800 μm by 200–300 μm by 20–50 μm and mounted between aluminium foil T-clips. Strips were then transferred to a mechanics workstation (Model 600, Aurora Scientific, Aurora, Canada) and mounted between a force transducer (Akers AE 801, MEMSCAP, San Jose, USA) and servomotor (Aurora Scientific, Aurora, Canada) in relaxing solution (pCa 9). Strips were stretched to just develop tension and then stretched to maximal length (L_o) (an additional 30%). Then the strips were skinned in pCa 9 with 1% Triton solution for 15–20 min, after which the smooth muscle strips were thiophosphorylated with a protocol consisting of bathing the fibre in rigor solution without Ca^{2+} (mM: 5 TES, 1.2 $MgCl_2$, 30 EGTA, 26 HDTA) for 10 min, followed by rigor with Ca^{2+} (mM: 50 TES, 1.2 $MgCl_2$, 28 Ca-EGTA, 1 EGTA, 26 HDTA) for another 10 min and then 30 min in rigor with Ca^{2+} and 10 mM ATPγS, then placed back in rigor solution. Tissue strips were then transferred to relaxing solution containing ATP (mM: 5.5 Na_2ATP, 56.5 potassium methane sulphonate, 7.2 $MgCl_2$, 25 creatine phosphate, 25 *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (Bes), and 0.02 $CaCl_2$, pH 7.0) and force increased to a steady-state plateau. Once reaching steady state, the maximal shortening velocity (V_{max}) was determined.

To determine shortening velocity, force was rapidly lowered by shortening the preparation and using a computer program to adjust muscle length to clamp force at a new isotonic level (50%, 30%, 20%, 10%, 5%, 1%). The velocity of shortening was defined as the linear fit between the times of 80 ms and 90 ms to the length *versus* time trace. The resulting data of force *versus* velocity was then fitted to the Hill equation $(V + b)(P + a) = b(P_o + a)$, where V is the rate of shortening, P is the clamped force, P_o is the maximal force (force at L_o), and a and b are constants. The maximal shortening velocity is extrapolated as the velocity at $P = 0$.

Mesenteric arteries. Mice were shipped to the University of Vermont and allowed to acclimate for 1 week prior to experimentation. They were then anaesthetized as described above and killed by cervical dislocation. The abdomen was opened by longitudinal incision, and a section of the gut and its vessels, approximately 5–10 cm distal to the pylorus, was excised and transferred to a Petri dish filled with ice-cold *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulphonic acid (Hepes)-buffered physiological saline solution (Hepes-PSS). Using microdissection tools, tertiary branches of the mesenteric arcade (unpressurized arterial diameter approximately 100 μm) were dissected free of surrounding connective and fat tissue and transferred to the experimental chamber of a small-artery arteriograph. Vessels were then tied onto two glass cannulas and pressurized to 50 mmHg using a pressure-servo apparatus. Changes in vessel diameter were measured with a video dimension analyser (Living Systems Instrumentation) and recorded on a PC computer using specialized imaging software. A more detailed description of the small artery pressure system can be found elsewhere (D'Angelo & Osol, 1993).

Following 60 min equilibration at 37°C, the vessels were constricted with a solution containing phenylephrine (10 μM) and a high concentration of potassium (124 mM) to induce maximal constriction. Time to half-maximal constriction ($T_{1/2}$, s), slope of maximal velocity during the initial phase of constriction (normalized to diameter by expressing the data as the percentage change in diameter per second), and the extent of maximal constriction (percentage reduction in lumen diameter) were determined from experimental records. Vessels were then subjected to step increases in transmural pressure in 20 mmHg increments until loss of constriction was observed (forced dilatation, FD, at pressures above 180 mmHg). The pressure at which FD occurred in each vessel was used to calculate average FD pressure (mmHg), thereby providing an index of the maximal force

production by vascular smooth muscle within the arterial wall.

Results

The double knockout mice lacking SM-B and h1-CaP do not show any gross abnormalities

We have previously shown that a complete switching from SM-B myosin to SM-A in the *smb*−/− mice resulted in decreased smooth muscle contractility, and an increased expression of h1-CaP and mitogen-activated protein kinase activity (Babu *et al.* 2001, 2004). To address the functional significance of h1-CaP up-regulation in the contractile properties of smooth muscles of SM-B null mice, we have generated a double knockout (dKO) mouse model for SM-B myosin and h1-CaP. The smooth muscle-specific h1-CaP knockout mouse model (Yoshikawa *et al.* 1998) maintained by Dr Katshuhito Takahashi, Japan was bred to the SM-B null mice (Babu *et al.* 2001). The heterozygous (*smb*+/− & *Cnn1*+/−) males and females were crossed to generate mouse models with genotypes of *smb*−/− and *Cnn1*+/−. The male and female *smb*−/− and *Cnn1*+/− mice were then bred together to generate homozygous (*smb*−/− and *Cnn*1−/−) dKO mice. Mice were genotyped by polymerase chain reaction as described earlier (Yoshikawa *et al.* 1998; Babu *et al.* 2001) to confirm the successful generation of dKO mice. The pups were delivered in a Mendelian ratio and reached adulthood without any gross morphological abnormalities, suggesting that loss of both SM-B myosin and h1-CaP did not affect smooth muscle growth and development and survival of the mice.

To verify that h1-CaP is not expressed in the smooth muscles of dKO mice, Western blot analysis was carried out using total homogenate from bladder and mesenteric vessels of wild-type (WT), *smb*−/− and dKO mice. Figure 1 shows that CaP expression was increased in the *smb*−/− null bladder (2.79 ± 0.10 fold; $P < 0.05$, $n = 3$, Fig. 1*A* and *C*) and in the mesenteric vessels (2.33 ± 0.03 fold; $P < 0.05$, $n = 3$, Fig. 1*B* and *C*) compared to their respective WT tissues as reported earlier (Babu *et al.* 2004), but was completely absent in the dKO mice. The expression level of non-muscle β-actin protein determined as internal loading control was unchanged between the groups. These results additionally confirm the successful generation of dKO mice lacking both SM-B and CaP proteins.

To determine if loss of h1-CaP expression alters the expression of other CaP isoforms, mRNA levels of neutral

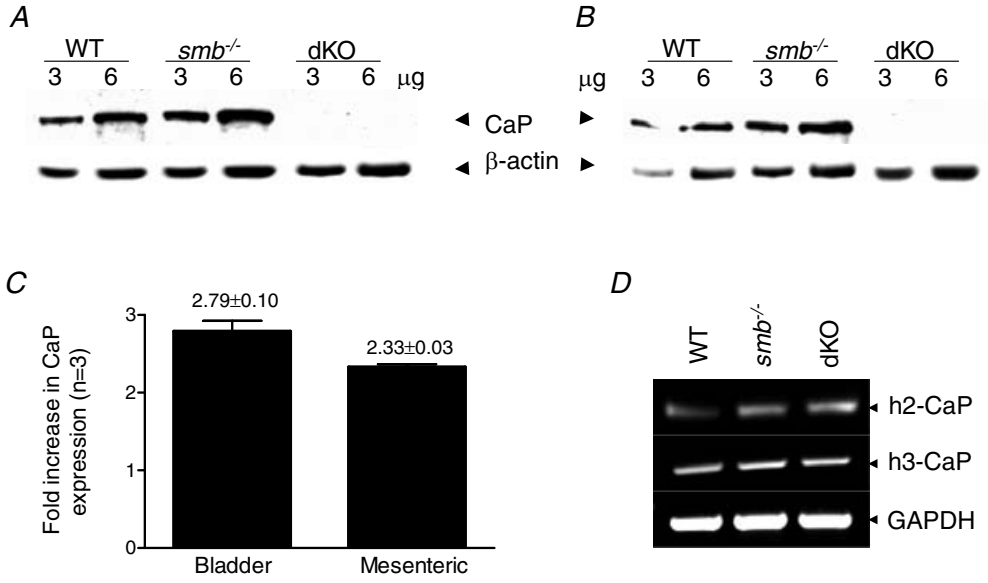

Figure 1. Calponin expression in SM-B null and dKO mice bladder
Two different concentrations (3 and 6 μg) of total protein extracted from wild-type (WT), *smb*−/− and double knockout (dKO) bladder (*A*) and mesenteric vessels (*B*) were separated on a 10% SDS-PAGE and analysed by Western blot analysis using anti-h1-calponin and β-actin antibodies. Results in *A* and *B* represent 3 independent experiments. *C*, bar graph shows the relative levels of CaP in the SM-B null bladder and mesenteric vessels compared to respective WT tissues. Calponin expression is increased over twofold in the SM-B null mice and completely abolished in the dKO mice. *D*, reverse transcription-polymerase chain reaction analysis of h2- and h3-CaP expression. Total RNA isolated from WT, *smb*−/− and dKO bladder tissues were used for the PCR analysis and GAPDH was used as internal control. Results in *D* represent 3 independent experiments. Expression levels of h2- and h3-CaP isoform mRNA levels are unaltered in the dKO bladder.

(h2)-CaP and acidic (h3)-CaP in bladder tissues were analysed by reverse-transcription-PCR. Our results shown in Fig. 1*D* demonstrate that loss of h1-CaP does not affect the expression of h2- or h3-CaP isoforms.

Loss of SM-B and h1-CaP did not affect the smooth muscle morphology

To determine whether loss of h1-CaP in SM-B null mice results in any structural abnormalities, histopathological analysis was carried out. Bladder tissues from *smb*−/− and dKO mice were fixed in 10% neutral buffered formalin, dehydrated through a gradient of alcohols, embedded in paraffin, and sectioned and stained with haematoxylin and eosin as described earlier (Karagiannis *et al.* 2003). Results in Fig. 2 show no significant difference between *smb*−/− and dKO mice bladder muscle. The smooth muscle morphology and orientation were normal and similar between single and dKO mice. These results suggest that loss of both SM-B myosin and h1-CaP did not alter smooth muscle structure and development.

Loss of SM-B and h1-CaP did not affect the myosin heavy and light chain isoform expression

To determine whether loss of both SM-B and h1-CaP proteins induced changes in myosin protein expression or isoform switching, Western blot analysis was carried out using protein extracts prepared from bladder and mesenteric vessels. Our results indicate that ablation of h1-CaP did not affect the level of SM1 (WT = 100%, *smb*−/− = 101 ± 2.2%, dKO = 99.8 ± 1.6%; $P > 0.05$), SM2 (WT = 100%, *smb*−/− = 104 ± 1.9%, dKO = 102 ± 1.6%; $P > 0.05$), LC_{17} (WT = 100%, *smb*−/− = 105 ± 3.0%, dKO = 101 ± 5.0%; $P > 0.05$) and LC_{20} (WT = 100%, *smb*−/− = 100 ± 4.6%, dKO = 103 ± 4.4%; $P > 0.05$) proteins in the dKO mice bladder (Fig. 3*A*). Similar results were also obtained with mesenteric vessels (Fig. 3*B*).

Smooth muscle α-actin levels are decreased in the dKO bladder

Western blot analysis revealed that smooth muscle α-actin levels are ∼40% down-regulated in the dKO bladder (Fig. 4*A* and *B*) and mesenteric vessels (Fig. 5*A* and *B*). These data are consistent with a previous report on the down-regulation of smooth muscle α-actin expression in h1-CaP single knockout bladder (Matthew *et al.* 2000). The γ-actin and non-muscle β-actin protein levels were unchanged in both bladder (Figs 1*A* and 4*A*) and mesenteric vessels (Figs 1*B* and 5*A*) of dKO mice, indicating that ablation of h1-CaP did not affect the expression of other actin isoforms.

Smooth muscle-specific h-CaD has been shown to be down-regulated in the *smb*−/− mice (Babu *et al.* 2004). To determine if loss of h1-CaP affects CaD levels, Western blot analysis was carried out using smooth muscle-specific h-CaD antibody. Our results show that h-CaD protein levels are decreased in the *smb*−/− bladder (Fig. 4*A* and *C*) and mesenteric vessels (Fig. 5*A* and *C*) as reported earlier (Babu *et al.* 2004). Interestingly, ablation of CaP restores the h-CaD expression in the dKO bladder (Fig. 4*A* and *C*) as well as in the mesenteric vessels (Fig. 5*A* and *C*) and its level is significantly higher than the wild-type tissues.

Contractile properties of dKO mice bladder

To determine how loss of CaP affects smooth muscle contractility in *smb*−/− bladder, we compared shortening velocity (V_{max}) and force generation between *smb*−/− and dKO mice bladder as described earlier (Karagiannis *et al.* 2003). Steady-state forces between WT and *smb*−/− bladder were not

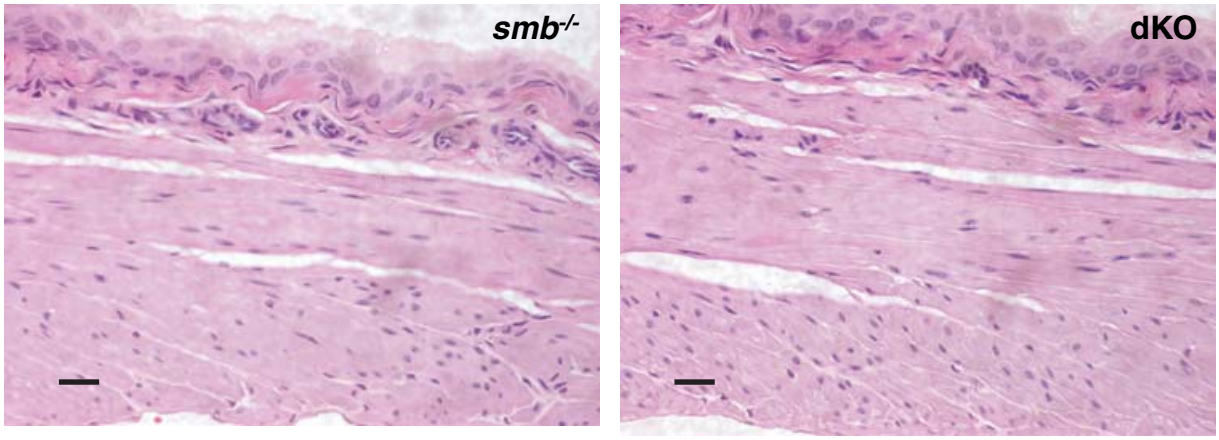

Figure 2. Histological staining of SM-B null and dKO mice bladder
Bladder tissue from *smb*−/− (left panel) and dKO mice (right panel) was fixed with formalin and stained with haematoxylin and eosin. Scale bar, 10 μm.

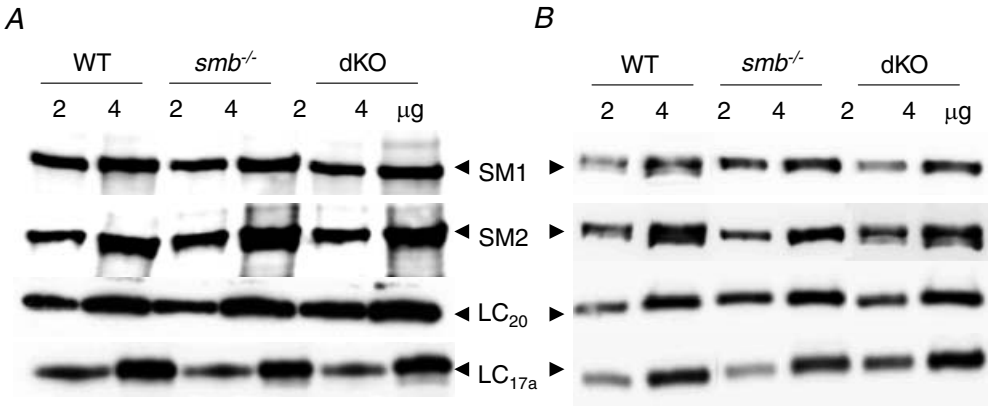

Figure 3. Expression of myosin heavy and light chain isoforms
Two different concentrations (2 and 4 μg) of total protein extracts prepared from *smb−/−* and dKO bladders (*A*) and mesenteric vessels (*B*) were separated on 5% (for SM1 and SM2) or 14% SDS-PAGE (for LC_{20} and LC_{17}) and immunoprobed with specific antibodies. Expression levels of myosin isoforms are unaltered in the dKO bladder. Signals were quantified by densitometry scanning. Data are representative of 3 independent experiments.

statistically different ($WT = 9.9 \pm 0.7\ mN\ mm^{-2}$ *versus* $smb-/- = 8.9 \pm 0.7\ mN\ mm^{-2}$); however, the steady-state force was significantly increased in the dKO bladder ($dKO = 12.5 \pm 0.5\ mN\ mm^{-2}$) and was significantly higher than WT (Fig. 6). As reported earlier (Karagiannis *et al.* 2003), the velocity of shortening was significantly decreased in the *smb−/−* bladder ($WT = 0.22 \pm 0.4\ ML\ s^{-1}$ $(n = 10)$ *versus* $smb-/- = 0.18 \pm 0.1$ muscle lengths (ML) s^{-1} $(n = 7)$). Interestingly, V_{max} in the dKO bladder ($dKO = 0.24 \pm 0.2$ $(n = 9)$) was no different to that of WT bladder, but significantly higher than that observed in the SM-B null mice.

Contractile properties of mesenteric vessels

As summarized in Table 1, there were no differences observed in all three groups in vessel diameter at 50 mmHg

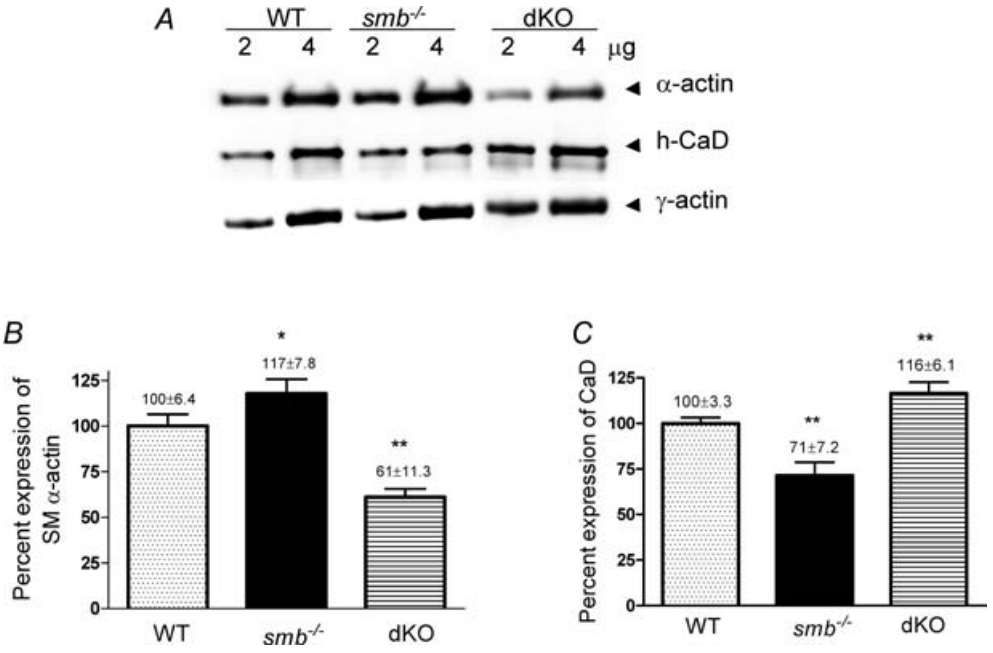

Figure 4. Expression of smooth muscle α-actin and h-CaD in dKO bladder
A, representative Western blots showing the expression of α-actin, γ-actin and h-CaD expression in dKO bladder. γ-Actin levels are unchanged between the three groups. *B* and *C*, bar graphs showing the percentage expression of smooth muscle α-actin and CaD proteins in the dKO bladder. Smooth muscle α-actin is down-regulated ~40% (*B*) whereas h-CaD is up-regulated (*C*) in the dKO bladder. *Not significantly different from WT. **Significantly different from the other two groups.

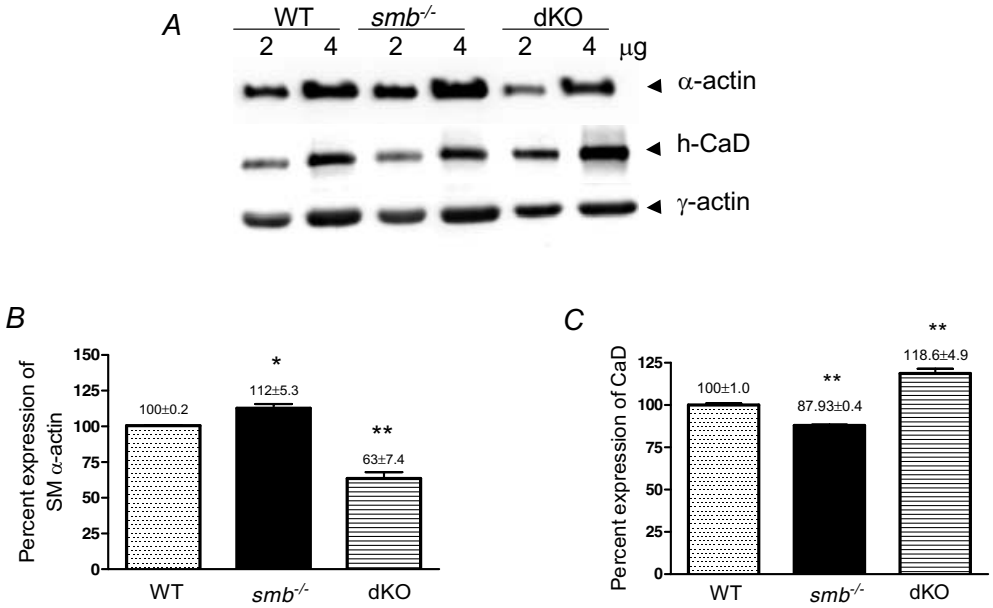

Figure 5. Expression of smooth muscle α-actin and h-CaD in dKO mesenteric vessels
A, representative Western blots showing the expression of α-actin, γ-actin and h-CaD expression in dKO mesenteric vessels. γ-Actin levels are unchanged between the three groups. *B* and *C*, bar graphs showing the percentage expression of smooth muscle α-actin and h-CaD proteins. Smooth muscle α-actin is down-regulated ∼40% (*B*) whereas h-CaD is up-regulated (*C*) in the dKO mesenteric vessels. *Not significantly different from WT. **Significantly different from other two groups.

in a relaxing solution containing papaverine (0.1 mM) in the extent of maximal vasoconstriction to an activation cocktail containing phenylephrine (10 μM) and potassium (120 mM) or in the equivalent maximal reductions in lumen diameter (approximately 80%). Half-time ($T_{1/2}$) to maximal constriction was significantly increased in both knockout (*smb*−/− and dKO) groups, indicating decreased shortening velocity, as was the normalized slope of constriction (percentage diameter change per second) in vessels from both the *smb*−/− and dKO animals (Table 1).

Relative to vessels from the *smb*−/− animals, the pressure at which forced dilatation occurred was significantly increased in the dKO mesenteric vessels, and reached the levels of WT control vessels (Table 1). Forced dilatation (FD; used in the present study) is an indirect measure of maximal force production, and is defined as the point at which transmural pressure overcomes the ability of vascular smooth muscle to produce an adequate counterforce to circumferential tension produced by the intraluminal pressure. These data showing enhanced force production by mesenteric artery smooth muscle from dKO *versus smb*−/− animals are in general agreement with those obtained from bladder smooth muscle and described above.

Discussion

The major goal of the current study was to determine the functional significance of h1-CaP up-regulation in

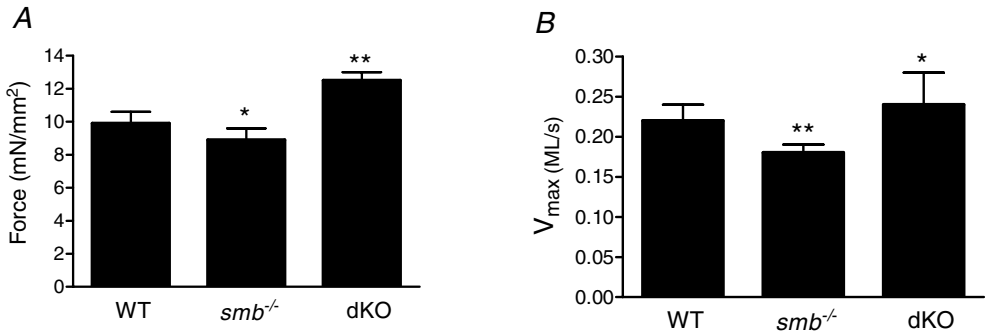

Figure 6. Functional measurements on *smb*−/− and dKO bladder
A, force; *B*, V_{max}. Data are means ± S.E.M. ($P < 0.05$). *Not significantly different from WT. **Significantly different from the other two groups.

Table 1. Contractile properties of mesenteric vessels

Experimental parameters	WT ($n = 5$)	*smb*−/− ($n = 6$)	dKO ($n = 5$)
Maximal relaxed lumen diameter at 50 mmHg (μm)	196 ± 17.6	198 ± 16.5	200 ± 10.3
Maximal constriction (% decrease in lumen diameter)	79 ± 3.3	80 ± 1.6	79 ± 1.5
Time to 50% constriction (s)	4.2 ± 0.49†	5.2 ± 0.68*	5.6 ± 0.58*
Slope of constriction (normalized: % change in lumen diameter s^{-1})	12.8 ± 1.45†	10.5 ± 1.33*	9.6 ± 1.37*
Forced dilatation pressure (mmHg)	204 ± 6.7†	183 ± 7.8*	202 ± 8.2†

Vessels were constricted with an activation cocktail containing phenylephrine (10 μM) and potassium (124 mM). Different symbols (†,*) indicate significant differences ($P < 0.05$) between groups; identical symbols are not significantly different from each other. All comparisons performed by two-way ANOVA.

SM-B null mice (Babu *et al.* 2004). To critically asses the role of h1-CaP in the contractile function of SM-B null smooth muscles, we ablated h1-CaP in the SM-B null background by crossing h1-CaP and SM-B single knockout mice. The key findings of this study are as follows. (1) Ablation of CaP in the SM-B null mice bladder resulted in increased V_{max} and steady-state force generation. (2) In mesenteric vessels, the maximal force as measured by forced dilatation was increased in the dKO mice, and the higher maximal force allows the vessel to shorten faster under a load. The shortening velocity, however, was decreased in both *smb*−/− and dKO vessels. (3) Interestingly, these functional changes were observed with a decrease in smooth muscle α-actin levels and an increase in caldesmon levels.

We have previously shown that switching of SM-B to SM-A myosin in a SM-B null mouse model resulted in decreased velocity of shortening (Babu *et al.* 2001; Karagiannis *et al.* 2003) and increased expression of h1-CaP (Babu *et al.* 2004). The available data strongly suggest that h1-CaP is involved in the regulation of actin–myosin interaction and therefore the kinetics of smooth muscle contractility (Takahashi & Yamamura, 2003). Ablation of h1-CaP significantly increases the unloaded shortening velocity in phasic smooth muscle tissues such as bladder and vas deferens (Matthew *et al.* 2000; Takahashi *et al.* 2000). These findings taken together suggest that the up-regulation of h1-calponin could negatively influence the velocity of shortening in SM-B null mice. Therefore, the dKO mouse model lacking both h1-CaP and SM-B myosin provide us with an excellent model to determine the functional significance of h1-CaP up-regulation in SM-B null mice. In dKO mice, loss of both h1-CaP and SM-B myosin proteins does not affect survival or cause any overt smooth muscle pathology as observed in the individual knockout animals (Yoshikawa *et al.* 1998; Babu *et al.* 2001). Loss of h1-CaP is not compensated by other CaP isoforms (Fig. 1*C*) and consistent with the previous studies on h1-CaP single knockout (Yoshikawa *et al.* 1998). Interestingly, loss of CaP in the SM-B null background is associated with ∼40% reduction in the smooth muscle α-actin levels. However, loss of h1-CaP does not affect the β- and γ-actin isoforms (Figs 1, 4 and 5). These changes are also consistent with changes associated with CaP single knockout mice (Matthew *et al.* 2000) and indicate the preservation of the CaP null phenotype in the dKO mice.

As previously reported, the loss of SM-B myosin does not alter the unitary force generation in bladder muscle compared to the WT bladder (Karagiannis *et al.* 2003, 2004). However, ablation of h1-CaP resulted in increased steady-state force generation in the SM-B null mice. Increased steady-state force generation under conditions where LC_{20} were maximally, irreversibly phosphorylated, and in the absence of structural changes (as observed by histology) suggests that h1-CaP may play a role in modulating bladder smooth muscle contractility. However, these results differ from the previous report using h1-CaP single knockout mice, which showed no significant difference in force development in the skinned bladder muscles between WT and h1-CaP null mice (Matthew *et al.* 2000), and rather decreased force production in intact vas deferens and aortic smooth muscles (Takahashi *et al.* 2000; Fujishige *et al.* 2002). The unexpected increase in force in the dKO bladder could be due to the complete replacement of SM-B by the SM-A myosin isoform in the bladder and/or other compensatory changes yet to be analysed.

In agreement with previous results, the WT bladder shortened at a rate 20% faster than the *smb*−/− bladder (Karagiannis *et al.* 2003). The V_{max}, however, was significantly increased in the dKO bladder as compared to *smb*−/− bladder, under conditions in which the levels of myosin thiophosphorylation were similar. These results indicate that myosin light chain phosphorylation does not contribute for the observed velocity changes. A similar increase in V_{max} has been reported for h1-CaP single knockout (Matthew *et al.* 2000; Takahashi *et al.* 2000). We have previously shown that the 7 amino acid insert regulates the earlier rate-limiting step including nucleotide binding and inorganic phosphate release (Karagiannis *et al.* 2003) whereas CaP is proposed to regulate the later steps in the cross-bridge cycle such as dissociation of high-affinity cross-bridges from actin, by binding to actin (EL-Mezgueldi & Marston, 1996; Takahashi & Yamamura, 2003). Therefore, both h1-CaP and SM-B myosin could

affect the cross-bridge cycling and V_{max} but at different kinetic steps. Taken together our studies suggest that up-regulation of h1-CaP in the SM-B null mice may be a compensatory alteration to maintain a reduced level of cross-bridge cycling over time in the absence of SM-B myosin. However, reduction in the smooth muscle α-actin levels and increase in h-CaD levels suggest that changes in other contractile and regulatory proteins may also contribute to the altered contractile properties observed in the dKO bladder.

In addition to bladder, the contractile properties of mesenteric vessels were determined in the dKO mice. We have previously reported that SM-B myosin is expressed heterogeneously in the mesenteric vessels and contributes to their unique mechanical properties (Babu *et al.* 2004). In that study, we also found that loss of SM-B myosin resulted in increased force generation under isometric conditions (using wire-mounted vessel rings) in response to phenylephrine + potassium (Babu *et al.* 2004). In the present study, which used a more physiological pressurized vessel approach, maximal constriction to an activation cocktail containing high potassium (124 mM) and phenylephrine (10 μM) was similar in all treatment groups. Since the vessel is studied as an intact unit, the maximal extent of constriction, measured as the reduction in lumen diameter from a fully relaxed state, may be limited by other factors, e.g. cell–matrix interactions, rather than simply maximal force generation.

The (1) increased half-time to maximal constriction, and (2) reduced normalized slope of constriction observed in mesenteric vessels of both *smb*−/− and dKO mouse models, both suggest decreased shortening velocity of smooth muscle. These findings are consistent with our previous studies showing decreased shortening velocity in the mesenteric vessels with SM-B deficiency (Babu *et al.* 2001, 2004), and indicate that changes in h1-CaP do not seem to be the underlying cause for the altered kinetics in this type of smooth muscle. Another study using rat portal vein also showed that the maximal velocity of vascular smooth muscle shortening was independent of h1-CaP expression (Facemire *et al.* 2000).

The significantly reduced forced dilatation pressure in vessels from the *smb*−/− but not dKO mice was an unexpected finding that suggests that the modulatory effect of calponin on vascular smooth muscle (VSM) contractility may be influenced by (1) the extent of VSM activation, (2) spatial events such as the degree of actin–myosin overlap (the length–tension relationship), and (3) the nature of the distending force acting upon the vascular wall. All three possibilities derive from our use of the more physiological pressurized vessel methodology in this study, since earlier work used the isometric wire-mounted approaches in which vessels are stretched to a fixed length prior to activation and therefore unable to shorten in response to activation. In the pressurized vessel, as in the body, activation results in constriction, which is a reflection of smooth muscle shortening. This may have implications for the spatial relationship between actin, myosin and calponin, and thereby alter the nature of thin filament regulation, as there were no detectable differences in myosin heavy and light chain isoform expression. Further, in pressurized vessels, the force experienced by the vascular wall is true transmural pressure, which has a radial as well as a circumferential component that may influence the distribution of intramural stress. Although it was beyond the scope of this study to differentiate the ultrastructural/spatial *versus* biomechanical components of the response, these elements deserve consideration in future studies. Finally, although the velocity data were consistent with previous findings in mesenteric vessels (Babu *et al.* 2004) and in bladder smooth muscle (this study), it should be noted that, in arteries, the importance of shortening velocity *in vivo* is questionable, while the production and maintenance of force is essential to the maintenance of vascular tone and, thereby, peripheral resistance, blood pressure and normal organ perfusion. This differs from the bladder, whose proper function is dependent on smooth muscle shortening for effective micturition. Thus, it is not unreasonable to expect differences in the nature of h1-CaP regulation based on physiological function. Based on the results of our studies, h1-CaP appears to be a key regulator of both bladder and vascular smooth muscle contractility, although its precise modulatory role may clearly vary with smooth muscle tissue type and function.

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Ackowledgements

This work was supported by American Heart Association Grant 0365173B (to G.J.B.), National Heart, Lung, and Blood Institute Grant HL-38355-17 (to M.P.) and HL-44181 (to F.V.B.).