Contractile properties and proteins of smooth muscles of a calponin knockout mouse

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1. The role of h1-calponin in regulating the contractile properties of smooth muscle was investigated in bladder and vas deferens of mice carrying a targeted mutation in both alleles designed to inactivate the basic calponin gene. These calponin knockout (KO) mice displayed no detectable h1-calponin in their smooth muscles.

2. The amplitudes of Ca^{2+} sensitization, force and Ca^{2+} sensitivity were not significantly different in permeabilized smooth muscle of KO compared with wild-type (WT) mice, nor were the delays in onset and half-times of Ca^{2+} sensitization, initiated by flash photolysis of caged GTPγS, different.

3. The unloaded shortening velocity (V_{us}) of thiophosphorylated fibres was significantly ($P < 0.05$) faster in the smooth muscle of KO than WT animals, but could be slowed by exogenous calponin to approximate WT levels; the concentration dependence of exogenous calponin slowing of V_{us} was proportional to its actomyosin binding *in situ*.

4. Actin expression was reduced by 25–50%, relative to that of myosin heavy chain, in smooth muscle of KO mice, without any change in the relative distribution of the actin isoforms.

5. We conclude that the faster V_{us} of smooth muscle of the KO mouse is consistent with, but does not prove without further study, physiological regulation of the crossbridge cycle by calponin. Our results show no detectable role of calponin in the signal transduction of the Ca^{2+}-sensitization pathways in smooth muscle.

Smooth muscle contraction is primarily regulated via reversible Ca^{2+}–calmodulin-dependent phosphorylation of myosin regulatory light chain (MLC_{20}; Hartshorne, 1987; Somlyo & Somlyo, 1994), but the existence of additional, thin filament-mediated regulatory mechanisms that modulate contractility has also been proposed (Marston & Smith, 1985; Walsh, 1991). Basic or h1-calponin, herein referred to as calponin, is a 34 kDa smooth muscle-specific, actin-associated protein first isolated from bovine aorta (Takahashi *et al.* 1988), and a putative candidate for mediating thin filament regulation of smooth muscle (Lehman, 1991; Winder *et al.* 1998). It is a specific marker of smooth muscle phenotype (Takahashi *et al.* 1987; Gimona *et al.* 1990; Samaha *et al.* 1996) and the major calponin isoform expressed in smooth muscle (Jin *et al.* 1996). Exogenous calponin inhibits both maximal shortening velocity (Jaworoski *et al.* 1995; Obara *et al.* 1996) and isometric tension (Itoh *et al.* 1994; Jaworoski *et al.* 1995; Uyamu *et al.* 1996) in permeabilized smooth muscle containing endogenous calponin and, *in vitro*, calponin inhibits actin-activated Mg^{2+}-ATPase activity (Takahashi *et al.* 1986; Winder & Walsh, 1990; Horiuchi & Chacko, 1991) and actin filament velocity (Shirinsky *et al.* 1992; Haeberle, 1994). It has been suggested that calponin maintains smooth muscle in a relaxed state at low/moderate levels of phosphorylated MLC_{20} (Malmqvist *et al.* 1997). However, conclusive evidence of its physiological role in smooth muscle is yet to be established and in a recent study comparing smooth muscle in rat strains with or without constitutive calponin expression, the only difference observed in smooth muscle lacking calponin was a small decrease in agonist sensitivity (Nigam *et al.* 1998), a change that could be due to a decrease in the Ca^{2+}-sensitizing effects of agonists. Ca^{2+} sensitization describes the processes by which agents can increase myosin phosphorylation and force independently of changes in $[Ca^{2+}]_i$ (Somlyo & Somlyo, 1994). Although the involvement of calponin in Ca^{2+} sensitization is unknown, based on an immunofluorescence study of the cellular redistribution of calponin, calponin has been implicated in protein kinase C (PKC)-mediated, Ca^{2+}-independent

Table 1. Probable derivation of structural genes with contractile function in WT and KO mice

Gene product	Gene symbol	Human mapping*	Mouse mapping†			Parental derivation§	
			Confirmed	Predicted‡	Ratio	WT	KO
Calponin	*Cnn1*	19p13.2	9:7.0 cM	—	—	B	A
Caldesmon	*Cald1*	7q33	6:11.5 cM	—	—	A	A
α-Actin	*Actvs*	10q22–q24	unknown	19:25.0–42.0 cM	4/8	A	A/B
				14:2.5–14.0 cM	3/8	B	B
				10:3.5 cM	1/8	A	A/B
β-Actin	*Actb*	7p22–p12	5:80.0 cM	—	—	A	A
γ-Actin	*Actg*	17q25	unknown	11:72.0–78.0 cM	7/7	A×B	BRA/B
α-Tropomyosin	*Tpm1*	15q22.1	9:40.0 cM	—	—	A	B
β-Tropomyosin	*Tpm2*	9p13.2–p13.1	unknown	4:12.7–20.8 cM	9/10	B	A
				11:26.0 cM	1/10	A	A

*Mapping data retrieved from Online Mendelian Inheritance in Man (OMIM; McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University, Baltimore, MD, USA) and National Center for Biotechnology Information (National Library of Medicine, Bethesda, MD, USA; May, 2000; World Wide Web URL http://www.ncbi.nlm.nih.gov/omim/). †Mapping data retrieved from the Mouse Genome Database (MGD; Blake *et al.* 2000; Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, ME, USA; May, 2000; World Wide Web URL http://www.informatics.jax.org/). cM, centimorgans. ‡Regions of conserved synteny in mouse derived from closely linked human genes with defined and mapped mouse orthologues (no. of mouse orthologues in the predicted region/no. of total mapped mouse orthologues to human genes linked to the primary locus). §Allele designations: A = 129X1/SvJ; B = C57BL/6; A/B = either A or B homozygote; A×B = heterozygote; R = recombinant.

contractions (Parker *et al.* 1994, 1998). A calponin gene-knockout strategy offers a powerful and unique means for testing the relative importance of calponin in the regulation of smooth muscle contraction. Therefore, in this study we compared Ca^{2+} sensitization, force, shortening velocity and the stoichiometry of contractile and regulatory proteins in smooth muscle from calponin KO and WT mice.

METHODS

Mice

A targeted mutation was induced in CCE ES cells (obtained from an inbred mouse line 129/SvJ; Gondo *et al.* 1994). F2 mice homozygous for the mutant basic calponin allele (KO) or the wild-type allele (WT) were generated after outcross to C57BL/6 mice as previously described (Yoshikawa *et al.* 1998). Homozygous mice of each genotype from the F2 generation were then used to derive independent KO and WT lines. The mice used for these experiments were taken from the F2 $N \geqslant 12$ generations. The absence of h1-calponin expression in the KO mice was confirmed by Western blotting (Fig. 1). Mice were killed by halothane or CO_2 inhalation, as approved by the Animal Research Committee of the University of Virginia.

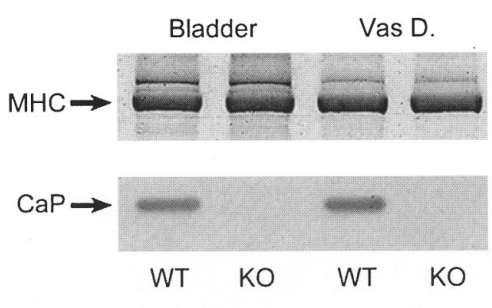

Figure 1. Western blot for calponin (CaP) showing its presence and absence in WT and KO mice, respectively

Similar protein loading is shown by Coomassie Blue staining of myosin heavy chain (MHC). Vas D., vas deferens.

Genome scanning

Using DNA prepared from one WT and five randomly selected KO mice, 73 mapped microsatellite loci (Dietrich, 1996; World Wide Web URL http://carbon.wi.mit.edu:8000/cgi-bin/mouse/index) previously determined to be polymorphic in a C57BL/6J × 129X1/SvJ (formerly 129/SvJ) cross were analysed using methods adapted from Dietrich *et al.* (1992). Briefly, PCR amplification from genomic DNA was performed using fluorochrome-labelled primers purchased from Research Genetics, Inc. (Huntsville, AL, USA) or synthesized from published oligonucleotide sequences (Integrated DNA Technologies, Coralville, IA, USA). Products from 10–16 loci per animal were pooled following amplification and analysed for allele sizes using an ABI377 automated sequencer equipped with Genescan v. 2.1 and Genotyper v. 2.0 software (PE Applied Biosystems, Foster City, CA, USA). Strain determination was made using control DNA from C57BL/6J, 129X1/SvJ and (C57BL/6J × 129X1/SvJ)F1 mice. Detailed amplification protocols, locus list and allele sizes are available on request (M. J. McDuffie; email: mjm7e@virginia.edu). Inheritance of specific loci relevant to contractile function in smooth muscle was inferred through the genotypes of flanking markers when their localization in the mouse genome was known or predictable based on regions of human/mouse conserved synteny (Table 1).

Analysis of contractile and regulatory proteins

Bladder or vas deferens smooth muscle tissue was cleaned of connective tissue, blotted and weighed before homogenization in sample buffer (2% SDS, 10 mM dithiothreitol, 10% glycerol, a trace amount of Bromophenol Blue and 50 mM Tris HCl, pH 7.4) to give a stock concentration of 10 mg ml^{-1} (wet w/v). After homogenization,

samples were heated at 85°C for 5 min and allowed to stand at room temperature for 60 min before centrifugation at 10 600 *g* for 10 min. Total protein extracts were subjected to SDS–PAGE and either stained with Coomassie Blue (0.2% w/v) or transferred to polyvinylidene fluoride membranes for subsequent Western blotting. For quantitative analysis of myosin heavy chain (MHC) and actin content, their relative concentrations were determined from serial dilutions of whole homogenate and compared to a linear range of bovine serum albumin standards, after correction for differential staining intensity with Coomassie Blue (Haeberle *et al.* 1992). The relative distribution of actin isoforms was determined by two-dimensional gel electrophoresis (2% ampholytes, pH 4–6.5) and staining with Coomassie Blue. Using densitometric analysis of Coomassie Blue-stained gels loaded with equal amounts of homogenate, the relative amounts of tropomyosin and h-caldesmon were expressed as a ratio of KO to WT. The identities of calponin, seven amino acid-inserted MHC, h-caldesmon and tropomyosin were confirmed by Western blotting. The calponin antibody was generated to a carboxyl-terminal peptide of calponin (Yoshikawa *et al.* 1998).

MLC_{20} thiophosphorylation

The levels of MLC_{20} thiophosphorylation were determined by one-dimensional isoelectric focusing (2% ampholytes, pH 4.5–5.4) and transfer to polyvinylidene fluoride membranes for subsequent Western blotting with an anti-MLC_{20} antibody.

Isometric tension measurement and solutions

Bundles of bladder muscle (3 mm × 200–400 μm) or strips of vas deferens (3 mm × 100–200 μm) were dissected from male mice (6–12 weeks, aged matched for WT and KO) and tied with monofilament silk to a fixed hook and force transducer (AE 801; AME, Horten, Norway) for measurement of isometric tension at room temperature (20°C) (Horiuti *et al.* 1989). For evaluating Ca^{2+} sensitization or relaxation from rigor, tissues were permeabilized with *Staphylococcus aureus* α-toxin (7500 rabbit units ml^{-1}) for 45 min. Otherwise, tissues were permeabilized with 0.5% Triton X-100 for 20 min. All intracellular solutions had an ionic strength of 0.2 M (adjusted with potassium methanesulfonate), and contained 30 mM Pipes (pH 7.1), 2 mM free Mg^{2+}, 4.5 mM MgATP, 5 mM creatine phosphate and 10 mM total EGTA (Ca-EGTA and EGTA were combined to produce the desired pCa) except for rigor solutions, which did not contain MgATP or creatine phosphate.

Ca^{2+} sensitization

After permeabilization with α-toxin, bladder bundles were incubated with A23187 (10 μM) for 10 min to deplete internal Ca^{2+} stores before equilibration in G10 solution (Ca^{2+} free, 10 mM EGTA) for 20 min. A steady-state pCa–force relationship was then obtained by cumulative addition of Ca^{2+}. After relaxation in G10, strips were equilibrated for a further 20 min before obtaining a Ca^{2+}-sensitization response in pCa 6.0 solution by cumulative addition of GTP (50 μM) and carbachol (100 μM), phorbol 12,13-dibutyrate (PDBu; 1 μM) or GTPγS (100 μM). After obtaining steady-state tension, strips were transferred to pCa 4.5 solution to elicit the maximal Ca^{2+}-activated tension.

In some experiments, the kinetics of GTPγS-induced Ca^{2+} sensitization were compared in smooth muscle from WT and KO animals using flash photolysis of caged GTPγS to circumvent diffusional delays (Somlyo & Somlyo, 1990). Briefly, α-toxin-permeabilized bundles of bladder were transferred from G1 (Ca^{2+}-free, 1 mM EGTA) to a 25 μl trough containing pCa 6.0 solution containing caged GTPγS (200 μM) and glutathione (3 mM) for 5 min. Caged GTPγS was photolysed using a frequency-doubled ruby laser (Lumonics, Warwick, UK), delivering a 50 ns pulse of near-UV light at 347 nm, sufficient to release 14 ± 2.3 μM GTPγS ($n = 6$, where n refers to the number of experimental observations), as determined by HPLC of the trough solution after completion of the force measurements. The delay between the laser flash and initiation of force by Ca^{2+} sensitization was measured from the point at which the force record started to deviate from the pre-photolysis baseline. The amplitude of the GTPγS-induced Ca^{2+} sensitization was measured from the pre-photolysis pCa 6.0-induced force plateau.

Determination of force per cross-sectional area

Bladder or vas deferens preparations were Triton permeabilized and washed for 15 min in G10 solution before activation in pCa 5.0 solution (containing 2 μM calmodulin) and subsequent relaxation in G10 solution. After tension had returned to baseline, the muscles were fixed whilst in the muscle trough, in 2% glutaraldehyde for 30 min, then cut down and transverse sections prepared (as detailed under 'Electron Microscopy'). Sections (1 μm thick) were stained with Toluidine Blue, mounted under a coverslip, and photographed at ×80 magnification. The mean cross-sectional area of each strip was determined from three sections taken at different levels. Each section area was developed onto 10 cm × 15 cm prints, counted using a transparent grid overlay and compared to prints of known area.

Electron microscopy

Strips of bladder or vas deferens were fixed in 2% glutaraldehyde in 75 mM sodium cacodylate buffer with 4% sucrose overnight at 4°C, followed by fixation in 2% osmium tetroxide and 1% tannic acid, *en bloc* staining with saturated uranyl acetate, dehydration in alcohol and embedment in Spurr's resin. Thin transverse sections were stained with lead citrate and examined in a Philips CM12 electron microscope at 80 keV (1 eV = 1.60219×10^{-19} J). Cells were evaluated for filament content and differences in morphology between the WT and KO specimens.

Unloaded shortening velocity

V_{us} was determined using the slack test method (Edman, 1979; Matthew *et al.* 1998). Briefly, Triton-permeabilized muscle preparations, attached to hooks via aluminium T-clips, were washed in zero-Ca^{2+} rigor solution for 15 min before transfer to ATP-free, pCa 5.0 (Ca^{2+}-rigor) solution containing 1 mM ATPγS and 2 μM calmodulin for 10 min. After thiophosphorylation, preparations were washed in zero-Ca^{2+} rigor solution for 15 min before activation of tension in G10 solution (plus 8 mM creatine phosphate and 100 units ml^{-1} creatine kinase). Upon reaching steady-state isometric tension, length changes of varying amplitude, 7–20% of the initial muscle length and sufficient to reduce tension to baseline, were applied to one end of the muscle strip using an isotonic lever (Model 308, Cambridge Technology, Watertown, MA, USA; maximum step time, 300 μs); the time taken for the strip to take up the slack (Fig. 2*A*) was plotted against the length change and the slope of this relationship was taken as the V_{us} (Fig. 2*B*). To minimize depletion of MgATP and accumulation of MgADP, trough solutions were changed every 3 min during determination of V_{us}.

In some experiments, the effect of exogenous calponin on the shortening velocity of vas deferens strips from KO mice was examined. Strips were thiophosphorylated as above, but also incubated for a further 30 min in calponin (0–12 μM)-containing rigor solution before activation of force. Before use, calponin was extensively dialysed against 20 mM Pipes (pH 7.1) and 1 mM DTT and rapidly frozen as a 2 mg ml^{-1} stock. Vials were thawed immediately before use.

Co-sedimentation of calponin with actomyosin

In order to determine whether there was a correlation between the effect of exogenous calponin on shortening velocity and calponin

binding to actomyosin, Triton-permeabilized strips of vas deferens from KO mice were incubated for 30 min in calponin (0–12 μM)-containing rigor solution, washed in calponin-free G10 solution (4.5 mM MgATP) for a further 30 min, lightly blotted and rapidly frozen in liquid N_2. Thawed strips were homogenized in 100 μl lysis buffer (1% Nonidet P-40, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 0.1 mM leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and 20 mM Tris HCl (pH 7.2). Cellular debris was pelleted at 800 *g* for 10 min. The supernatant was transferred to an ultracentrifuge tube and spun at 200 000 *g* for 30 min at 20°C (TLA 120.1, Ultima TLX, Beckman) to sediment actin and myosin. The pellet and supernatant (1/3 total volume) were resuspended in 2× Laemmli sample buffer and analysed using one-dimensional SDS–PAGE to determine whether calponin distributed with actomyosin or was retained in the supernatant. To test whether calponin itself precipitated under these conditions, 100 μl lysis buffer containing 800 ng calponin was used as a control.

Relaxation from rigor

The apparent second-order rate constants of ATP-induced crossbridge detachment from high tension rigor, in the absence of Ca^{2+}, were estimated in α-toxin-permeabilized vas deferens from WT and KO mice as previously described (Fuglsang *et al.* 1993; Khromov *et al.* 1996). Briefly, after establishment of high-tension rigor, strips were incubated for 3 min with caged ATP before photolytic release of ATP. The ensuing relaxation consisted of an initial fast component and a second, slower component. The rate constant of the fast component was determined over a range of ATP concentrations (50–400 μM). Fitting of this relationship provided an estimate of the apparent second-order rate constant of MgATP-induced crossbridge detachment and was performed using SigmaPlot 4.0 software (Jandel Scientific, San Rafael, CA, USA).

Materials

All reagents were obtained from Sigma Chemical Co. except for α-toxin, which was purified from *Staphylococcus aureus*, A23187 (Calbiochem, La Jolla, CA, USA), ATPγS and GTPγS (Boehringer Mannheim, Indianapolis, IN, USA), caged GTPγS (a generous gift from Drs D. Trentham and G. Reid), pH ampholytes (Pharmalyte, Pharmacia, Piscataway, NJ, USA), chicken gizzard calponin (a generous gift from Dr M. Walsh), anti-MHC insert antibody (a generous gift from Dr A. Rovner), anti-myosin essential light chain (MLC_{17}) antibody (a generous gift from Dr K. Trybus), anti-smooth muscle myosin phosphatase regulatory subunit antibody (a generous gift from Dr D. Hartshorne), anti-MLC_{20} antibody (a generous gift from Dr K. Kamm), HRP-conjugated anti-mouse antibody (Goldmark Biologicals, Philipsburg, NJ, USA) and HRP-conjugated anti-rabbit antibody (Amersham, Piscataway, NJ, USA).

Statistics and data analysis

Data are presented as the mean ± S.E.M. and statistical significance was determined using Student's *t* test for single comparisons or Student's *t* test with the Bonferroni modification for multiple comparisons. The force records from the flash photolysis and slack

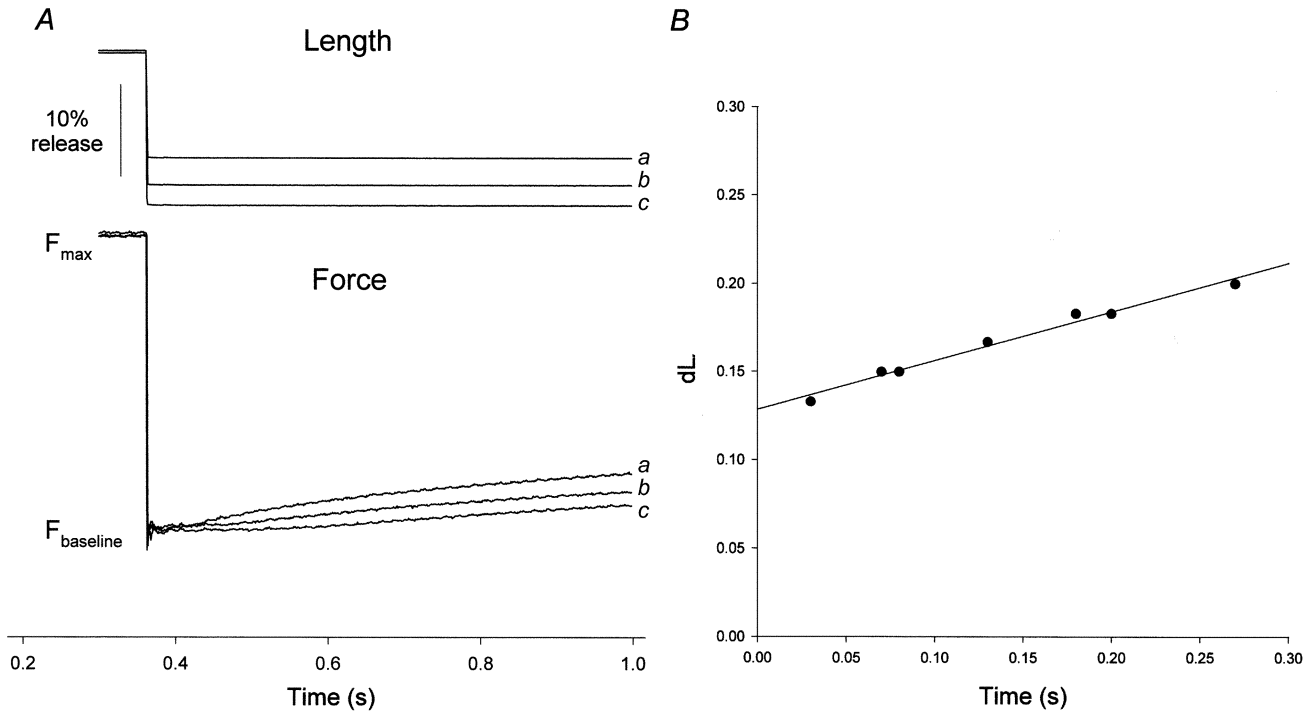

Figure 2. Measurement of unloaded shortening velocity

A, superimposed original force traces from mouse bladder smooth muscle demonstrating the slack test. After thiophosphorylation, steady-state force was obtained in the presence of 4.5 mM MgATP (F_{max}). Slack was introduced by imposing rapid releases up to ~20% of the initial muscle length. The length step was kept constant and the time for the imposed slack to be taken up by the muscle was indicated by the deviation from $F_{baseline}$ of the force tracing. Note that the shorter the length step, the shorter the time to remove slack. *B*, plot of individual slack test measurements performed on the same strip. Note the linear relationship of dL *vs.* dt over this time range (where dL is length change, normalised to initial length). The slope of this relationship was taken as the unloaded shortening velocity.

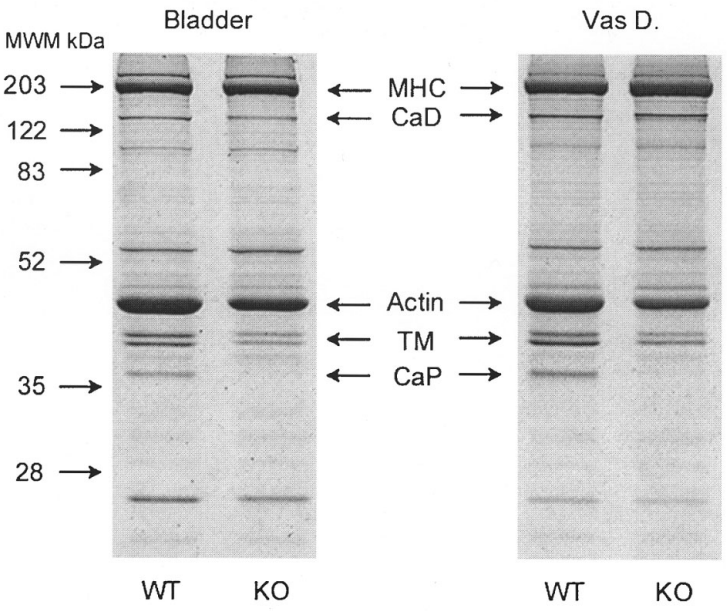

Figure 3. Coomassie Blue stain of smooth muscle protein extracts from WT and KO mice using 12% SDS–PAGE

Positions of major contractile proteins are indicated. Note that in extracts from calponin KO mice, there is a concomitant decrease in actin and tropomyosin (TM) relative to myosin heavy chain (MHC). The identities of calponin (CaP), TM and caldesmon (CaD) were confirmed by Western blotting. The size of the protein bands (in kDa) is indicated beside the gel.

test experiments were collected using LabView 3.1.1 data acquisition software (National Instruments, Austin, TX, USA). Quantification of protein bands was determined by gel-scanning densitometry (GS 670, BioRad, Hercules, CA, USA). All gels and Western blots presented are representative of at least three similar experiments.

RESULTS

Mice

As originally described (Yoshikawa *et al.* 1998), the calponin KO animals did not differ from WT mice in terms of body weight, growth, fertility or activity and the offspring of heterozygote matings exhibited Mendelian distribution.

Contractile and regulatory protein content

Initial experiments were conducted to determine whether, in addition to inactivation of the calponin gene (Fig. 1), the expression of other contractile or regulatory proteins was also altered. In smooth muscle from KO mice, the amount of MHC was not significantly different from WT (Fig. 3 and Table 2), nor was any change detectable in the relative expressions of the seven amino acid-inserted MHC isoform (Fig. 4*A*), MLC_{17} isoforms (Fig. 4*B*), myosin light chain kinase (Fig. 5*A*), or the regulatory subunit of smooth muscle myosin phosphatase (Fig. 5*B*). However, the amount of actin was significantly ($P < 0.05$) reduced by ~25% in the bladder and ~50% in

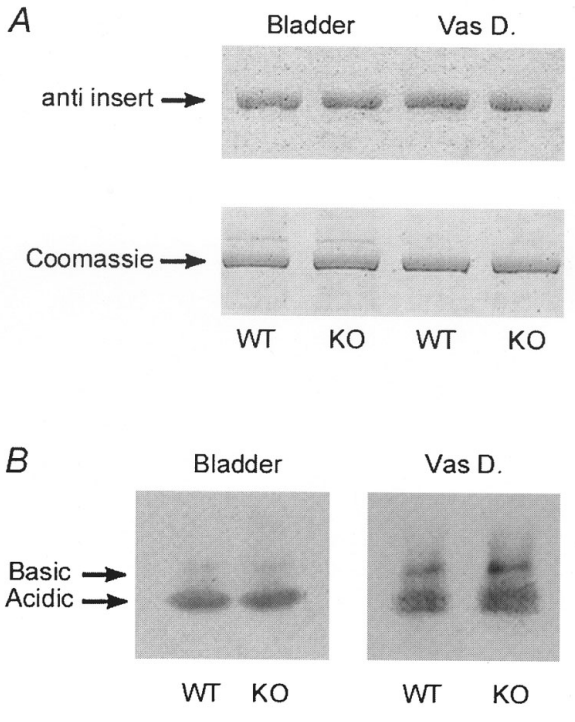

Figure 4. Expression of myosin isoforms in WT and KO tissue

A, Western blot for the 7 amino acid-inserted isoform of MHC (anti insert, upper panel) using 10% SDS–PAGE. Relative loading is indicated by the Coomassie Blue stain (lower panel) of MHC remaining in the gel after transfer. *B*, Western blot for myosin essential light chain (MLC_{17}) acidic and basic isoforms using one-dimensional isoelectric focusing.

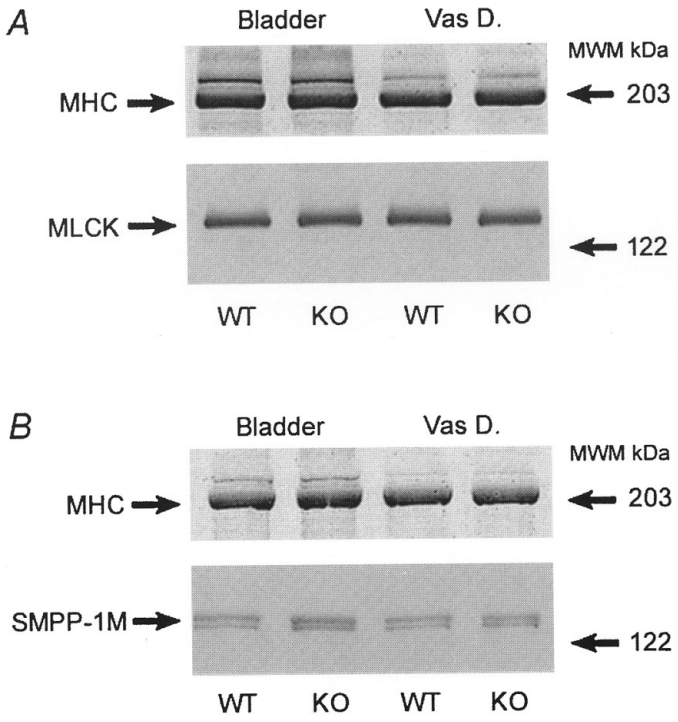

Figure 5. Expression of MLCK and SMPP-1M in WT and KO tissue using 10% SDS–PAGE

A, Western blot for myosin light chain kinase (MLCK). *B*, Western blot for the regulatory subunit of smooth muscle myosin phosphatase (SMPP-1M). In both *A* and *B*, relative loading is indicated by the Coomassie Blue stain for myosin heavy chain (MHC).

the vas deferens (Fig. 3 and Table 2). Using densitometric analysis, the KO/WT ratio of tropomyosin (α and β) expression was 0.5 ± 0.06 in the bladder ($n = 7$) and 0.5 ± 0.05 in the vas deferens ($n = 4$). The KO/WT ratio of h-caldesmon was 0.9 ± 0.03 in the bladder ($n = 6$) and 1.1 ± 0.05 in the vas deferens ($n = 5$) but, interestingly, an upwards shift in the SDS–PAGE mobility of h-caldesmon was present in both KO smooth muscles (Fig. 6).

Assuming molecular masses of 41 kDa for actin and 203 kDa for MHC, the approximate actin:MHC molar ratio was reduced from 16:1 to 10.5:1 in the bladder and from 12:1 to 5.2:1 in the vas deferens of KO animals compared to WT. This reduction in total actin was not associated with any change in the relative distribution of the α, β and γ isoforms of actin (Table 3), but was paralleled by a reduction in actin filament density (see 'Morphology' below).

Ca^{2+} sensitization

Neither the Ca^{2+} sensitivity nor the relative amplitude of the responses to the Ca^{2+}-sensitizing agents carbachol, PDBu or GTPγS (Fig. 7) was significantly different between α-toxin-permeabilized bladder preparations from WT and KO mice. The pCa–tension curves were superimposable with pCa for half-maximal response (pCa_{50}) values of 5.5 ± 0.06 (WT) and 5.5 ± 0.08 (KO) (both $n = 6$). In order to test whether the kinetics of GTPγS-induced Ca^{2+} sensitization were affected in the KO mouse, GTPγS was released by flash photolysis of caged GTPγS at constant [Ca^{2+}] (Fig. 8). No significant difference was observed in the delay, half-time or relative amplitude of contraction between muscles from WT and KO animals (Table 4).

Unloaded shortening velocity

V_{us} was significantly faster ($P < 0.05$) in KO compared to WT preparations in both the vas deferens and bladder

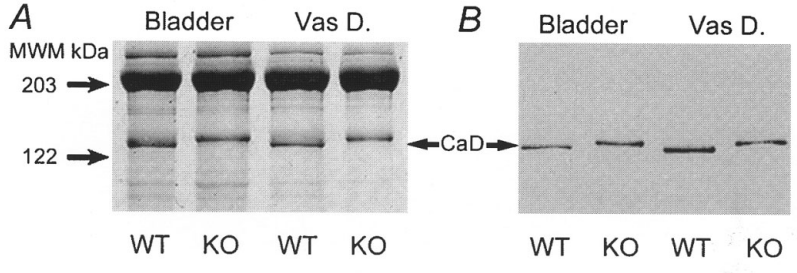

Figure 6. Expression of h-caldesmon in WT and KO tissue using 7.5% SDS–PAGE

A, Coomassie Blue stain showing an upwards shift in mobility of h-caldesmon (CaD) in KO tissue. The identity of CaD was confirmed by Western blotting (*B*).

Table 2. Quantitative analysis of myosin heavy chain and actin expression

Tissue	n	Myosin		Actin	
		WT	KO	WT	KO
Bladder	7	12 ± 1.6	14 ± 1.8	41 ± 3.3	29 ± 2.7*
Vas deferens	6	16 ± 1.1	19 ± 1.6	41 ± 3.4	20 ± 1.9*

Mouse bladder or vas deferens tissue was prepared, analysed using 10 or 12% SDS–PAGE, and quantified as described in Methods. Amounts of protein are expressed in micrograms per milligram tissue (wet wt). *$P < 0.05$ compared to WT tissue.

Table 3. Distribution of actin isoforms

Strain	n	Bladder			Vas deferens		
		α	β	γ	α	β	γ
WT	3	44 ± 3.5	10 ± 1.1	46 ± 2.3	49 ± 1.9	10 ± 2.1	41 ± 2.1
KO	3	44 ± 1.6	8 ± 1.1	47 ± 1.3	49 ± 3.3	11 ± 2.9	40 ± 0.7

Mouse bladder or vas deferens tissue was prepared, analysed using 2-D SDS–PAGE, and quantified as described in Methods. Values are expressed as a percentage of the total actin.

Table 4. Rate and amplitude of Ca^{2+} sensitization initiated by laser flash photolysis of caged GTPγS

Strain	n	Delay (s)	$t_{50\%}$ (s)	Percentage of pCa 4.5-induced tension
WT	10	4.3 ± 0.3	68 ± 4.8	52 ± 4.6
KO	9	4.8 ± 0.4	66 ± 7.1	57 ± 5.1

Mouse bladder bundles, α-toxin-permeabilized, were incubated with caged GTPγS (200 μM) for 5 min in pCa 6.0 solution. Force development by Ca^{2+} sensitization was initiated by laser flash photolysis as described in Methods. $t_{50\%}$, time to reach 50% of maximal GTPγS-induced Ca^{2+} sensitization.

(Table 5), whereas the levels of MLC_{20} thiophosphorylation in WT and KO mice were similar (vas deferens, 71 ± 4% for WT *vs.* 70 ± 2% for KO; bladder, 80 ± 3% for WT *vs.* 81 ± 2% for KO; all $n = 3$).

Force per cross-sectional area

No significant difference in the force per cross-sectional area was observed between Triton-permeabilized WT and KO muscles activated in pCa 5.0 solution. In vas deferens,

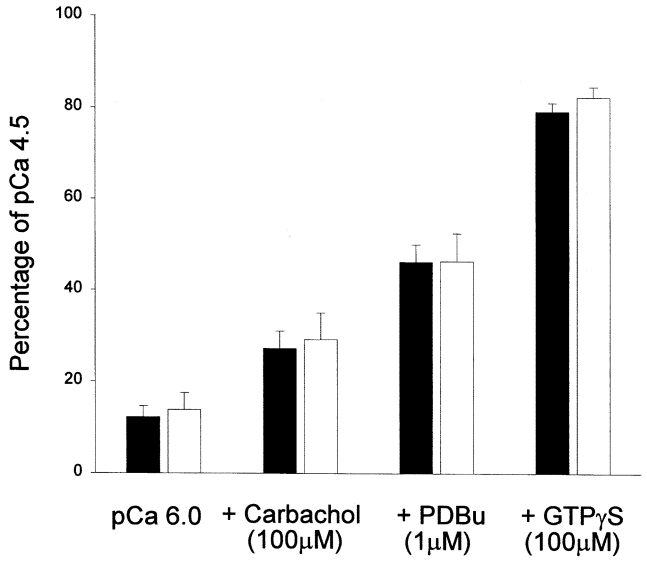

Figure 7. Summary of cumulative tension responses of α-toxin-permeabilized bundles of mouse bladder to Ca^{2+}-sensitizing agents

Tension is expressed as a percentage of the maximal force response to pCa 4.5. WT, ■ ($n = 6$); KO, □ ($n = 6$). Force in pCa 4.5 was 0.79 ± 0.10 mN for WT and 0.81 ± 0.11 mN for KO. Note that the different agents were added sequentially to the same strips and the cumulative response is shown.

Table 5. Unloaded shortening velocity (V_{us}) of Triton-permeabilized muscles

Tissue	WT	KO
Vas deferens	0.23 ± 0.01 (10)	0.31 ± 0.03 (8)*
Bladder	0.25 ± 0.03 (5)	0.36 ± 0.03 (7)*

After permeabilization, MLC_{20} was thiophosphorylated before activation of tension in G10 solution and measurement of V_{us} as described in Methods. Values are in muscle lengths per second; n is shown in parentheses. *$P < 0.05$, significantly different from WT.

the values were 18 ± 5.3 mN mm^{-2} for WT and 18 ± 5.2 mN mm^{-2} for KO (both $n = 4$), and for bladder they were 25 ± 6.1 mN mm^{-2} for WT and 19 ± 1.0 mN mm^{-2} for KO (both $n = 3$).

The effect of exogenous calponin on shortening velocity and lack of effect on force

To determine whether the above increase in V_{us} of muscles from the KO mice could be reversed by exogenous calponin, we incubated Triton-permeabilized, thiophosphorylated strips of vas deferens with increasing concentrations of calponin (0–12 μM). A concentration-dependent reduction in V_{us} (Fig. 9*A*) was shown to be proportional to the amount of calponin bound to actomyosin *in situ* (Fig. 9*B*). Note that no calponin was detectable in the supernatants (1/3 of the total supernatant volume was loaded), indicating that the majority of calponin retained after 30 min washing was bound to actomyosin. Although calponin (12 μM) caused a significant reduction in V_{us}, it had no significant effect on the amplitude of the force produced upon addition of 4.5 mM MgATP (after Triton permeabilization and MLC_{20} thiophosphorylation), expressed as a percentage of the initial response to high K^+ (154 mM) before permeabilization: 49 ± 12% in the 0 μM (dialysate buffer only) group and 54 ± 7% in the 12 μM calponin-treated group (both $n = 4$).

Relaxation from rigor

To determine whether the altered V_{us} in the KO mice was associated with a difference in nucleotide binding, we estimated the apparent second-order rate constant of MgATP-induced crossbridge detachment from rigor. There was no significant difference in the dependence on [MgATP] (Fig. 10) or the amplitude of the initial, rapid phase of relaxation from rigor between vas deferens strips from WT or KO mice. The apparent second-order rate constant, estimated using pooled data from the WT and KO mice, was 9.7 (± 0.6) × 10^4 M^{-1} s^{-1}, which is similar to the value obtained for phasic smooth muscle of the rabbit (Khromov *et al.* 1996).

Morphology

The ultrastructure was examined in vas deferens preparations from three calponin KO and two WT mice, in the portal anterior mesenteric vein of one KO and one WT mouse, and in the bladder from one KO mouse. All preparations were permeabilized with α-toxin, except for one pair of KO and WT vas deferens tissues, which were permeabilized with Triton X-100 prior to fixation. Permeabilization enhances the penetration of the fixative for improved preservation of the contractile proteins. Low magnification transverse views across the wall of the vas deferens or portal vein showed no obvious differences in wall thickness, cell shape or size, or in the size of the extracellular space. At higher magnifications in the α-toxin-permeabilized preparations, the mitochondria, cytoplasmic and membrane-associated dense bodies, endoplasmic reticulum and sarcoplasmic reticulum appeared normal in all tissues examined from both the WT and KO mice. In transverse sections, a regular distribution of myosin filaments occurred throughout the cytoplasm in all of the well-preserved preparations

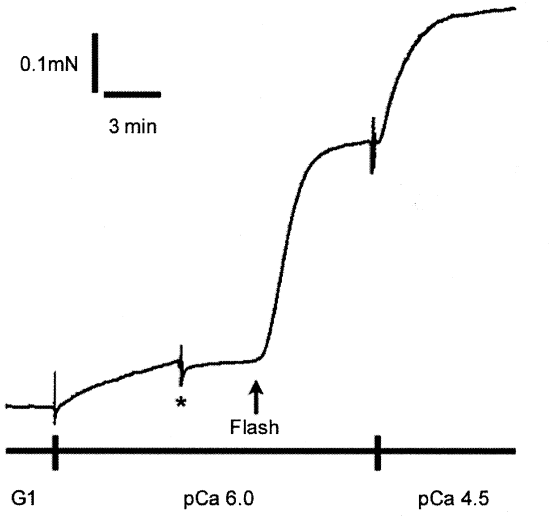

Figure 8. Representative tension trace illustrating the protocol for measuring the rate of Ca^{2+} sensitization induced by flash photolytic release of GTPγS from caged GTPγS in α-toxin-permeabilized mouse bladder

Sub-maximal tension was produced by transfer from G1 to pCa 6.0 solution. At the point indicated by the asterisk, the preparation was transferred from pCa 6.0 solution to the photolysis trough containing pCa 6.0 and 200 μM caged GTPγS. After approximately 3 min, laser flash photolysis of the caged compound released GTPγS, which produced an increase in tension. At the tension plateau, the preparation was transferred to pCa 4.5 solution to elicit maximal tension.

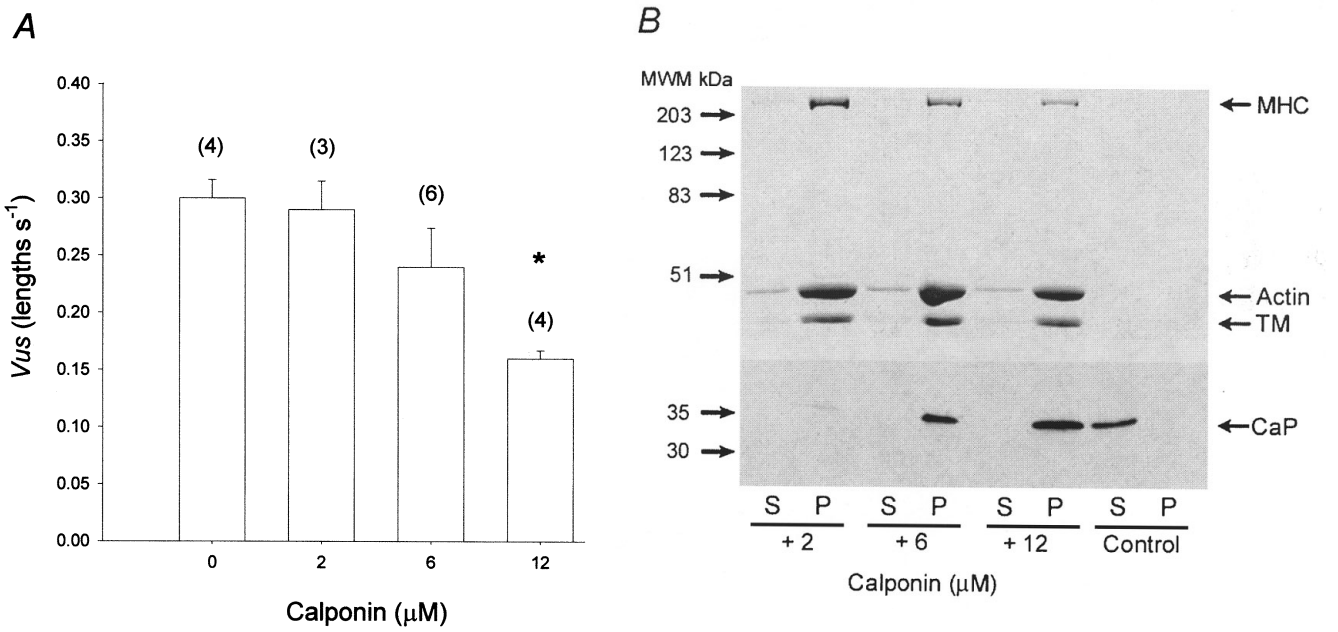

Figure 9. Inhibition of shortening velocity by exogenous calponin is porportional to its actomyosin binding *in situ*

A, exogenous calponin inhibits the shortening velocity of MLC_{20}-thiophosphorylated strips of vas deferens from KO mice. Strips of Triton-permeabilized KO vas deferens were thiophosphorylated and then incubated for a further 30 min with 0, 2, 6 or 12 μM calponin as described in Methods, before activation of force and measurement of shortening velocity. n for each concentration is shown in parentheses. * $P < 0.05$ compared to 0 μM. *B*, co-precipitation of exogenous calponin with native actomyosin from KO vas deferens, using 10% SDS–PAGE. Strips of Triton-permeabilized vas deferens from KO mice were incubated with 2, 6 or 12 μM calponin as described in Methods. Following high-speed centrifugation (200 000 *g*) of the tissue homogenate, the supernatant (S) and pellet (P) were analysed using 10% SDS–PAGE. The upper section of the gel, stained with Coomassie Blue, shows that the majority of the myosin heavy chain (MHC), actin and tropomyosin (TM) was located in the pellet. The lower section of the gel was transferred to polyvinylidene fluoride membrane and blotted for calponin (CaP). Note the similar concentration dependence of the co-precipitation of calponin with actomyosin and its effects on shortening velocity (compare with *A*). Control refers to high-speed centrifugation of exogenous calponin (800 ng) in the absence of tissue homogenate.

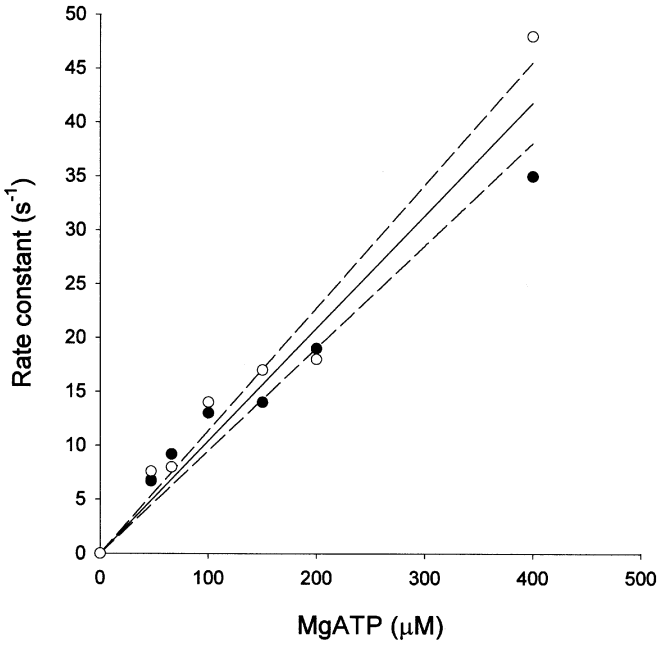

Figure 10. Concentration dependence of the rate constant of MgATP-induced crossbridge detachment from rigor

Strips of α-toxin-permeabilized vas deferens were prepared as described in Methods. Open and filled symbols represent WT and KO tissue, respectively. Each point represents a single observation. Fitting of the pooled data (continuous line) estimated a second-order rate constant of 9.7 ($\pm$ 0.6) $\times$ 10^4 M^{-1} s^{-1}. The dashed lines represent the 95% confidence limits.

(Fig. 11). To determine the structural correlates of the 51% decrease in actin determined by SDS–PAGE in the vas deferens from the KO mice, the ratio of actin to myosin filaments was measured in transverse sections in the same Triton-permeabilized muscle strips that were used to measure the total force per cross-sectional area. These muscles were set at the same resting length, and following the maximal Ca^{2+} contraction were relaxed to the resting level and fixed in this state. In micrographs at ×66 000 magnification, regions of transversely oriented actin and myosin filaments suitable for counting were circled and the number of actin and myosin profiles counted. The measurements were performed blind. The actin-to-myosin ratio in 28 regions from the vas deferens of the WT mouse was 11.3 ± 0.59, and was 6.6 ± 0.31 in 15 regions from the vas deferens of the KO mouse. The decrease in actin was highly significant ($P < 0.00001$).

DISCUSSION

The major findings of our comparison of smooth muscle of calponin KO with WT mice were (1) a faster V_{us} of KO fibres in which MLC_{20} was thiophosphorylated for maximal activation, an effect that could be inhibited by exogenous calponin, (2) altered expression of contractile proteins other than calponin, and (3) no difference in Ca^{2+}-activated tension or the rate constant of MgATP binding to crossbridges. The calponin KO mice developed normally and did not display increased morbidity or mortality compared with WT mice. In addition, unstimulated muscles of KO mice were not contracted, as might be expected if calponin was required to maintain smooth muscle in a relaxed state (Malmqvist *et al.* 1997). The basal level (in G10 solution) of MLC_{20} phosphorylation was ~10% (data not shown) in both WT and KO permeabilized bladder smooth muscle. In an earlier study of osteogenesis in this KO mouse, Yoshikawa *et al.* (1998) found no enhanced or ectopic expression of neutral or acidic calponin isoforms. Although neutral (Nigam *et al.* 1998) and acidic (Applegate *et al.* 1994) isoforms have been detected in some smooth muscles, their relative paucity of expression compared to basic calponin renders any compensatory effect of these isoforms on smooth muscle function highly unlikely. Previous *in situ* studies were limited to adding exogenous calponin to smooth muscle either expressing native levels of calponin (Itoh *et al.* 1994; Jaworoski *et al.* 1995; Obara *et al.* 1996; Uyama *et al.* 1996) or from which calponin had been non-selectively extracted (Malmqvist *et al.* 1997), whereas the knockout strategy provided us with a more direct method for examining the functional importance of calponin in smooth muscle regulation.

The V_{us} of KO smooth muscle was faster under conditions in which the level of MLC_{20} thiophosphorylation in WT and KO tissues was comparable and, therefore, the faster velocity of KO muscle is the result of a difference in crossbridge cycling, rather than the level of activation. The relative expressions of the seven amino acid-inserted MHC and the acidic MLC_{17} isoform were also unchanged,

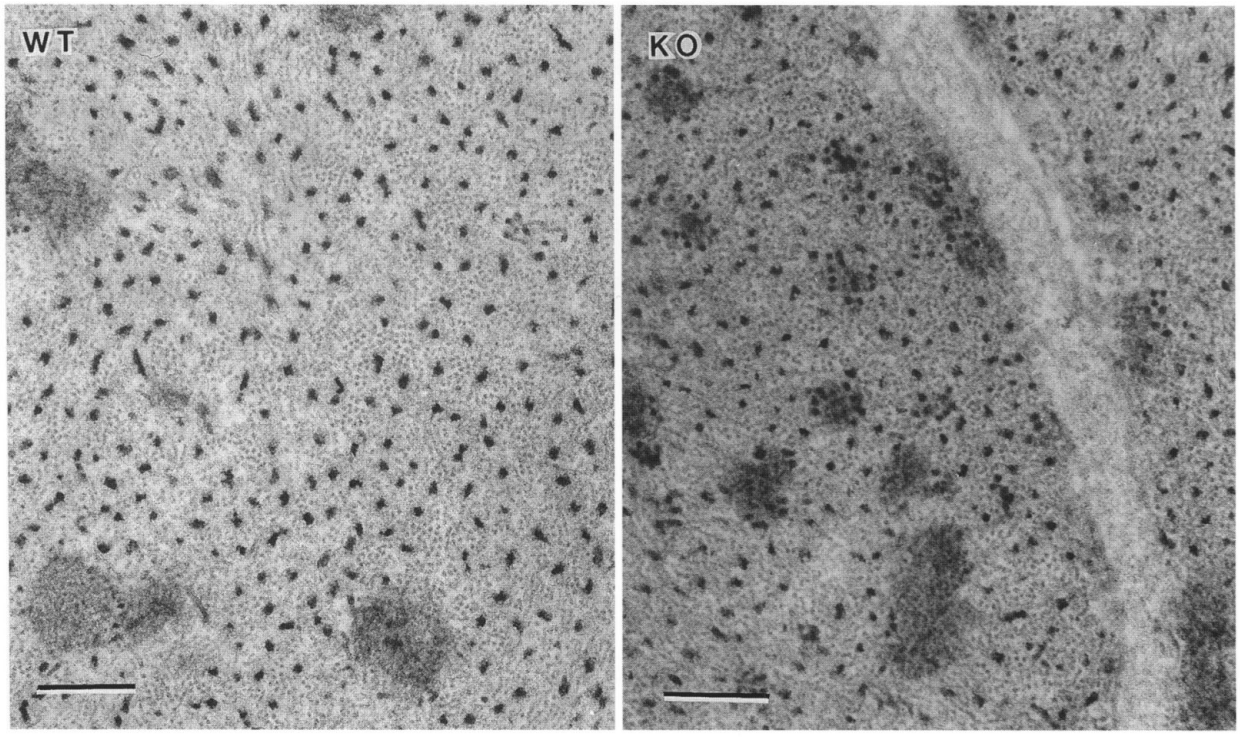

Figure 11. Electron micrographs of Triton-permeabilized WT and KO vas deferens

The cytoplasmic distribution of thick and thin filaments in transverse section. Scale bars represent 0.2 μm.

both of which correlate with faster shortening velocities in smooth muscle (Sjuve *et al.* 1996; DiSanto *et al.* 1997; Malmqvist & Arner, 1991; Matthew *et al.* 1998; reviewed in Somlyo, 1993). Consistent with the faster V_{us} in the absence of calponin, exogenous calponin inhibited the V_{us} of KO fibres to approximate WT levels without, however, a discernible effect on force. A selective effect of calponin on V_{us}, but not force, is supported by the similar Ca^{2+}-activated tension generated by smooth muscle from WT and KO mice, and is in agreement with studies in which exogenous calponin, added to permeabilized smooth muscle, affected velocity to a greater extent than force (Jaworoski *et al.* 1995; Obara *et al.* 1996). This implies a greater effect of calponin on g than f, the apparent rate constants for crossbridge detachment and attachment, respectively, described in detail by Huxley (1957). Since there was no difference in the apparent second-order rate constant of MgATP binding to crossbridges, the V_{us} differences in WT and KO tissue are likely to be rate limited by a later step(s) in the crossbridge cycle, other than MgATP binding, e.g. isomerization, inorganic phosphate (P_i) release, MgADP dissociation.

The shortening velocity of muscle is related to the number of contractile units in series ('minisarcomeres' in smooth muscle; Ashton *et al.* 1975), as well as to the crossbridge detachment rate (Rüegg, 1971). The apparent V_{us} of KO smooth muscle, when expressed in muscle lengths per second, may well be faster compared to WT if the reduced actin results in shorter minisarcomeres acting in series. The reduced number of actin filaments in electron micrographs of KO smooth muscles could be indicative of shorter minisarcomeres, but these measurements do not distinguish between a decreased length and a decreased number of filaments. Therefore, the possibility cannot be excluded that the decrease in actin, rather than the absence of calponin, is responsible for the faster V_{us} in smooth muscle of KO mice. *In vitro* (Kake *et al.* 1995), calponin has been shown to promote and stabilize actin polymerization, suggesting that actin filament formation, but not necessarily actin content, may be affected by the absence of calponin. That the faster V_{us} of KO mice can be reduced by exogenous calponin supports the hypothesis that calponin exerts a regulatory influence on the crossbridge cycle, probably mediated via its binding to actin (Takahashi *et al.* 1986; Winder & Walsh, 1990; Mezgueldi *et al.* 1992), although the co-precipitation result does not preclude binding also to myosin (Szymanski & Tao, 1993) or tropomyosin (Takahashi *et al.* 1987). Further, because exogenous calponin also reduces the V_{us} of smooth muscles containing a normal complement of endogenous calponin (Jaworowski *et al.* 1995; Obara *et al.* 1996), no claim can be made for a specific 'rescue' of KO smooth muscles by exogenous calponin.

The altered expression of actin and tropomyosin and the mobility shift of caldesmon in the calponin KO mouse were unexpected. They may reflect the co-ordinate regulation of calponin expression with that of other thin filament proteins, although the variations in genetic background of the WT and KO strains could also be responsible. A limited DNA microsatellite mapping study was performed to address the issue of genetic background. Consistent with the number of intercross generations, 85–90% of the loci were typed as homozygous in the WT and KO mice. Forty per cent of the homozygous loci in the KO mice were C57BL/6J derived, compared with 45% in the WT animal. The excess of 129 alleles in KO mice was caused by the expected selective retention of a large fraction of chromosome 9 surrounding the putative calponin locus (NCBI LocusLink; Maglott, 2000; World Wide Web URL http://www.ncbi.nlm.nih.gov/genome/sts/sts.cgi?uid=125918). The five KO mice were essentially concordant for parental derivation except in regions where persistent heterozygosity was detected (~15% of loci). Based on genotypes at flanking markers, the regions encoding structural genes relevant to smooth muscle contractile function were tested for parental derivation in KO and WT mice. With the exception of the expected discordance found near the calponin gene, only the intervals flanking the tropomyosin α and β loci (Table 1) were clearly dissimilar in parental derivation. Genome scan data, however, demonstrated that the WT and KO mice differed across intervals on 13 chromosomes, including most of chromosomes 1 and 9 (Table 1 and data not shown). Furthermore, five loci carried a third, unidentified allele suggesting genetic contamination of one or both of the parental strains (D5Mit240, D7Mit78, D8Mit112, D10Mit35, D12Nds2). Thus, although allelic differences in trans-acting regulatory genes are unlikely to result in the co-ordinate decrease in actin subunit expression or the mobility shift seen in the caldesmon protein from KO mice, such trans-acting gene effects cannot be ruled out until congenic mice with defined, equivalent genetic backgrounds are available.

The different SDS–PAGE mobility of h-caldesmon in WT and KO tissues resembles a previously reported h-caldesmon doublet (Bretscher, 1984) attributable to a heterogeneity of exon expression (Payne *et al.* 1995). Whether the shift in h-caldesmon mobility (Fig. 6) is of the same origin, its functional significance, if any, is unknown.

Consistent with observations on other mammalian smooth muscle (Kitazawa *et al.* 1989; reviewed in Somlyo & Somlyo, 2000), a Ca^{2+}-sensitizing mechanism was present in the mouse and unequal maximal responses (Himpens *et al.* 1990) could be elicited by carbachol, PDBu or GTPγS in α-toxin-permeabilized bladder. Since the responses to these stimuli were additive, it is likely that distinct G-protein- and PKC-coupled pathways of Ca^{2+} sensitization can be activated in this tissue (Jensen *et al.* 1996). The amplitudes of Ca^{2+} sensitization produced by these agents were not different in WT and KO preparations (Fig. 7), nor were the delay in the onset and

the rate of GTPγS-induced Ca^{2+} sensitization following flash photolysis of caged GTPγS (Table 4). These findings imply that the Rho-A signalling pathway leading to inhibition of myosin phosphatase (reviewed in Somlyo & Somlyo, 2000) is not altered in the muscles from KO animals. Assuming no compensatory mechanism masking the effects of the absence of calponin, we conclude that calponin is not necessary for signal transduction of G-protein- or phorbol ester-mediated pathways of Ca^{2+} sensitization in mouse smooth muscle. This is in agreement with a calponin-independent pathway of PKC-induced Ca^{2+} sensitization in rabbit vascular smooth muscle (Kitazawa *et al.* 1999), but does not support a cellular redistribution of calponin being a necessary step in PKC-dependent contraction of smooth muscle (Parker *et al.* 1998).

In conclusion, the present results are consistent with, but do not prove, a physiological role for calponin in regulating the crossbridge cycle in smooth muscle. In addition, the absence of calponin was without effect on force, Ca^{2+} sensitivity or Ca^{2+} sensitization in the mouse smooth muscles studied.

Note added in proof

An increase in shortening velocity in intact, non-permeabilized smooth muscle of calponin knockout mice has been noted in a preliminary report by K. Takahashi, M. Mitsui-Saito, K. Fuchibe, Y. Yamamura, S. Taniguchi, M. Katsuki, H. Ozaki, T. Tsuchiya, N. Shibata & H. Karaki (*Biophysical Journal* **74**, A38 (1998)) in which, however, the level of activation (intracellular Ca^{2+} and myosin light chain phosphorylation) was not controlled.

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