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# Regulation of Shortening Velocity by Calponin in Intact Contracting Smooth Muscles

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**To elucidate the function of calponin in intact contracting smooth muscle cells in vivo, we generated mice with a mutated basic calponin (h1) locus (Yoshikawa *et al., Genes Cells* 3, 685–695, 1998). Crossbridge cycling rates were estimated in aortic smooth muscle by the force redevelopment following an isometric step shortening as a function of time after $K^+$ depolarization. Evidence is presented that calponin is involved in the inhibition of shortening velocity in the tonic phase of contraction. The phosphorylation levels of myosin regulatory light chain and cytosolic calcium concentrations were not significantly different in paired comparisons between calponin-deficient (−/−) and wild-type (+/+) muscles at any time point after stimulation. The force-velocity relationships in vas deferens smooth muscle showed that the maximum shortening velocity of −/− muscle was significantly faster than that of +/+ muscle. There was no change in the length-force relationships in both −/− and +/+ muscles of aorta and vas deferens. The results suggest that calponin plays a role in regulation of the crossbridge cycling and that it may be responsible for reduced shortening velocity during a maintained contraction of mammalian smooth muscle.** 

*Key Words:* **calponin; knockout mice; smooth muscle; latch bridge; myosin light chain phosphorylation; calcium.**

Smooth muscle cells surrounding hollow organs do not work during tonic contractions to withstand continuous imposed loads such as blood pressure. In 1967, Somlyo and Somlyo described a catch-like behavior of vascular smooth muscle contraction (1). They found that when a quick release was applied shortly after the stimulus, the tension lost was recovered, whereas force did not redevelop when a quick release was applied in the tonic phase of contraction. Murphy and his colleagues demonstrated that both the maximum shortening velocity and myosin light chain phosphorylation attain maximal values early in the contraction and then decline, remaining at low levels in the tonic phase of contraction (latch-bridge) (2). These observations together with the energetic studies, demonstrating the 100 to 500 fold lower rate of ATP utilization per isometric force of smooth muscle than that of mammalian skeletal muscles (3), indicate that both force and velocity are regulated variables in smooth muscle contraction (4, 5). There is now substantial evidence indicating that reversible $Ca^{2+}$/calmodulin-dependent phosphorylation of myosin regulatory light chain primarily regulates the rate of ATP hydrolysis of smooth muscle myosin (6). In intact smooth muscle strips, however, it has been shown that isotonic shortening velocity and myosin ATPase activity can change independent of myosin light chain phosphorylation (7–9). The identification of molecular elements acting to maintain developed force with reduced crossbridge cycling rate in intact smooth muscle has been a subject of much research but remains a matter of controversy.

Calponin is a smooth muscle-specific and actin-associated protein first isolated from bovine aorta and chicken gizzard (10, 11). The three dimensional recon-

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struction of the actin-calponin structure demonstrates that calponin appears to contact with actin near both the N-terminus and residues close to the C-terminus (12). It has been suggested that the interaction of calponin with the glutamate residue at the C-terminus of actin molecule inhibits weak to strong transition of the actin-myosin binding, and thus play a role in the reduction of crossbridge cycling rate (13). Exogenous calponin inhibits both maximum shortening velocity (14, 15) and isometric tension (16–18) in permeabilized smooth muscle. Using an *in vitro* motility assay in combination with a method for estimating relative changes in the force exerted on actin filaments, Haeberle suggests that calponin inhibits the rate of dissociation of the high affinity actomyosin complex and, consequently, that it may be an integral component of the latch-state (19). On the other hand, using isolated single smooth muscle cell preparations, Malmqvist *et al.* have reported that calponin predominantly inhibits unphosphorylated crossbridges, and thus plays a role in maintaining a relaxed state and in inhibiting the formation of the latch-state (20).

To test the two seemingly contradictory hypotheses, we generated mice lacking smooth muscle calponin. In the present study, we performed mechanical measurements on intact smooth muscle strips obtained from aorta and vas deferens.

## MATERIALS AND METHODS

*Mice, antibodies, and immunoblot analysis.* Mice with a homozygous mutation in basic calponin (h1) allele were generated, and an antibody specific to basic calponin isoform was prepared as described previously (21). A monoclonal antibody against smooth muscle $\alpha$-actin (clone No. 1A4) was purchased from Sigma Chemicals (St. Louis, MO). Anti-h-caldesmon antibody was kindly provided by Dr. Mabuchi (Boston Biomedical Research Institute). The specificity of the antibody to each antigen was verified by the immunoblot analysis with an alkaline phosphatase staining (Bio-Rad Lab., Hercules, CA) as described previously (22).

*RNA preparation and reverse transcription PCR analysis.* Total RNA was extracted from aortic and vas deferens smooth muscle tissues, and subjected to the reverse transcription-PCR analysis as described previously (21). The parameters used for the PCR amplification were 30 cycles of denaturation (94°C, 40 s), annealing (60°C, 30 s) and polymerization (72°C, 90 s). Sequences of the selected forward and reverse 5′-to-3′ primers used, and predicted products size were as follows: SM1/SM2, AGGCAGAGGAGGAGTCCCAG (forward), ACTGGCTTTGGTTCCATTGA (reverse), 251 bp for SM1 and 290 bp for SM2 (23); G3PDH, CCCATCACCATCTTCCAGGA (forward), TTGTCATACCAGGAAATGAGC (reverse), 731 bp.

*Immunohistochemistry.* The specimens of aortic and vas deferens smooth muscles were rinsed with PBS and mounted in O.C.T. compound (Miles Inc., Elkhart, U.S.A.), and then frozen using liquid nitrogen. The frozen sections (4 $\mu$m in thickness) were immunostained with the monoclonal anti-$\alpha$-smooth muscle actin antibody (clone No. 1A4) or the polyclonal anti-basic calponin antibody according to the methods described previously (22).

*Electron microscopy.* Strips of 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, 1% tannic acid en bloc staining with saturated uranyl acetate, dehydration in alcohol, and embedment in Spurr's resin (24). Thin sections were stained with lead citrate and examined in a Philips CM12 electron microscope at 80 KeV.

*Measurements in muscle mechanics.* Thoracic aorta and vas deferens were isolated from 4∼7-week old male mice after sacrificing by rapid neck disarticulation and were cut into ring preparations in aorta (0.8–1.2 mm in width) and longitudinal strips in vas deferens (5–7 mm in length, 1–1.5 mm in width) under stereomicroscope. The Ringer solution had the following composition (mM): NaCl, 136.9; KCl, 5.4; $CaCl_2$, 1.5; $MgCl_2$, 1.0; $NaHCO_3$, 23.8; EDTA (ethylenediamine-*N,N,N′,N′*-tetraacetic acid) 0.01; glucose, 5.5. The solution containing 72.7 or 140 mM KCl for the stimulation of aorta and vas deferens, respectively, was made by substituting NaCl with equimolar KCl in the Ringer solution. These solutions were saturated with 95% $O_2$ and 5% $CO_2$ mixture at 37°C and pH 7.4. The aorta ring or the vas deferens strip with small connectors of aluminum foil at both ends was mounted on the two hooks from the servo-motor and the tension transducer. The preparation was equilibrated for 45–60 min in a 10 ml bath until the contractile response to high $K^+$ solution became stable. The aorta ring was stretched to an optimal length of the tissue for tension development. Tension change was measured by a transducer (AE801, Sensonor) with a compliance of 0.1 mm/N and a resonant frequency of about 5 kHz or by the feedback signal from servo-motor (Dual Mode Servo 300, Cambridge Technology). The tension and length changes were recorded with a digital oscilloscope (AR1200, Yokokawa or type 310, Nicolet). The force redevelopment after a length step in aorta was measured by imposing about 1.0% shortening steps. The shortening velocity in vas deferens was measured at 0.2 s after the isotonic release at the top of the tension development at various relative load and the force–velocity relationships in ordinary form and linear one, from which the maximum velocity was calculated, was obtained (25, 26).

*Measurement of myosin light chain phosphorylation.* Levels of myosin light chain phosphorylation were determined as reported by Word *et al.* (27). Strips of aorta and vas deferens were quickly frozen in liquid nitrogen and kept in acetone-dry ice containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT). After strips were homogenized in 10% TCA and 10 mM DTT, the homogenate was centrifuged at 10,000*g* and the pellet was washed with diethyl ether to remove TCA. The pellet was then suspended in urea-glycerol buffer for electrophoretic analysis of myosin light chain phosphorylation. Samples were loaded on polyacrylamide gels (10%) containing glycerol (40%) and electrophoresed for 90 min at 300 V. Protein was transferred to PVDF membrane at 2 mA/cm$^2$. Nonphosphorylated and phosphorylated form of myosin light chain on membrane were visualized by anti-bovine myosin light chain (generous gift from Dr. Stull) and anti-rabbit IgG from goat. Relative amount of nonphosphorylated and phosphorylated myosin were quantified by densitometric intensity using NIH Image.

*Measurement of cytosolic $Ca^{2+}$ level.* Cytosolic $Ca^{2+}$ was measured according to the method described previously (28), using fura-PE3 (Teflab, Austin, USA). Muscle strips were exposed to fura-PE3/AM (10 $\mu$M) in the presence of 0.02% cremphore EL for 6 h at room temperature. The muscle strips were transferred to the muscle bath integrated in the fluorimeter (CAF 110, JASCO, Tokyo) and illuminated alternately (48 Hz) with two excitation wave lengths (340 nm, 380 nm). Fluorescence at 500 nm was measured and the concentration of cytosolic $Ca^{2+}$ was calculated with an assumption of $K_d$ of $Ca^{2+}$ and fura-PE3 as 290 nM (29).

*Statistical analysis.* Statistical differences were determined by using the unpaired Student *t* test. Differences were considered statistically significant with $P < 0.05$.

## RESULTS

*Protein and mRNA expression.* On immunoblot analysis using a specific antibody raised against the

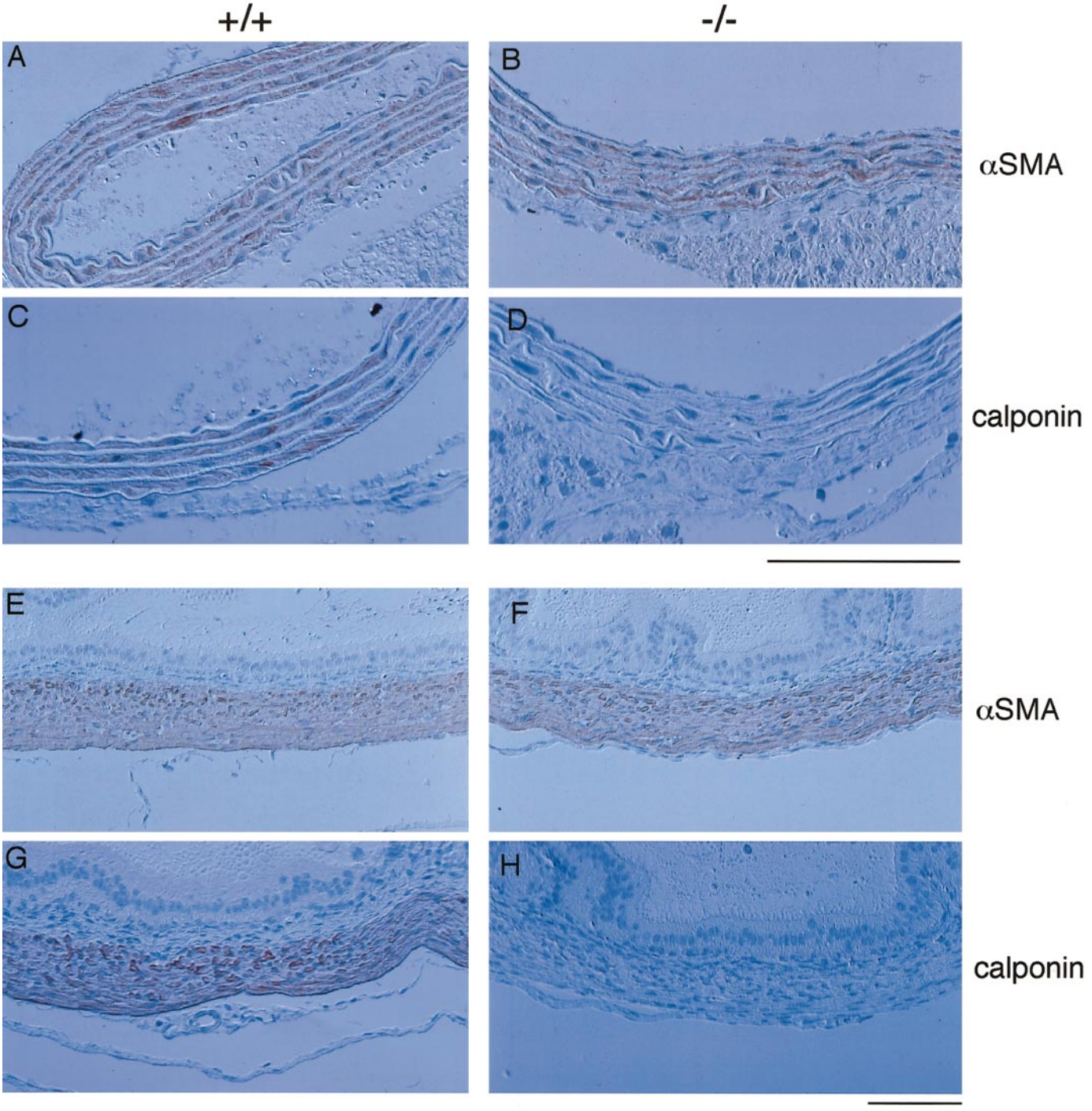


**FIG. 1.** Expression of calponin and $\alpha$-smooth muscle actin in aortic and vas deferens smooth muscles detected by immunohistochemistry. Serial sections of frozen smooth muscles from aorta (A–D) and vas deferens (E–G) were labeled with a monoclonal antibody to smooth muscle $\alpha$-actin (A, B and E, F) and a polyclonal antibody raised against basic isoform of calponin (C, D and G, H). Note that the loss of expression of calponin in $-/-$ mice (B, D, F, H) apparently did not affect the number of cell layers, cell shape or size in smooth muscle layers of aorta and vas deferens as compared with those prepared from $+/+$ mice (A, C, E, G). The scale bar represents 100 $\mu$m.

synthetic polypeptide of basic calponin (21), neither normal sized calponin protein nor any other fragments were detected in smooth muscle cells of aorta, vas deferens and stomach from $-/-$ mice, confirming the disruption of the calponin molecule (data not shown). At the mRNA level there were no compensations by non-smooth muscle isoforms of calponin. In addition, expression levels of h-caldesmon and $\alpha$-smooth muscle

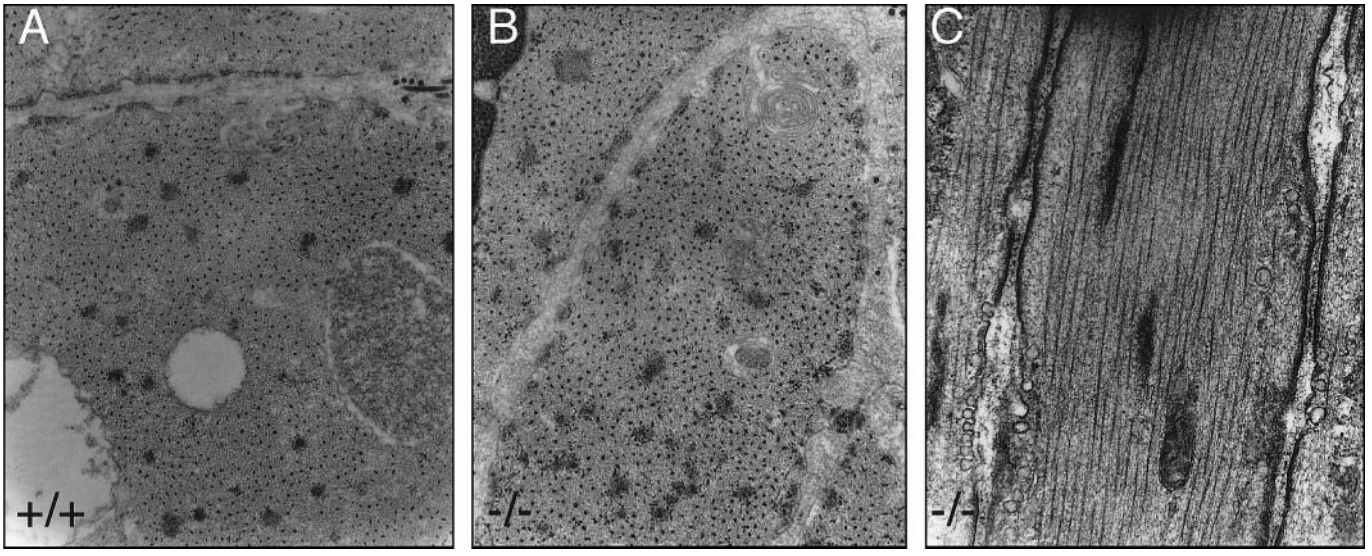


**FIG. 2.** Electron micrographs of transverse sections of Triton-permeabilized muscles (A, B) and a longitudinal section of α-toxin-permeabilized muscle (C) prepared from vas deferens of +/+ and −/− mice. Original magnification: ×66,000.

actin were not different between +/+ and −/− muscles. Furthermore, reverse transcription-PCR analysis demonstrated that expression levels of myosin heavy chain isoforms SM1 and SM2 were not different between +/+ and −/− muscles.

*Morphology.* Immunohistochemistry of aortic and vas deferens smooth muscle tissues obtained from 4 week-old +/+ and −/− mice probed with antibodies specific for α-smooth muscle actin or basic isoform of calponin showed that at light microscopic level, there appears no obvious differences in wall thickness, the number of cell layers, cell shape or size, and in the amount of extracellular matrix (Fig. 1). The ultrastructure was examined in vas deferens preparations permeabilized with α-toxin or 0.5% Triton X-100 (Fig. 2). In both transverse and longitudinal sections, a regular distribution of myosin filaments surrounded by actin filaments occurred throughout the cytoplasm in all of the well-preserved preparations from both +/+ and −/− mice. At higher magnifications in the α-toxin-permeabilized preparations (Fig. 2C), the mitochondria, cytoplasmic and membrane-associated dense bodies, endoplasmic reticulum and sarcoplasmic reticulum, and the size of the extracellular space appeared normal in all tissues examined from −/− mice.

*Crossbridge cycling in aortic smooth muscle.* No significant difference in the isometric force production per cross-sectional area was observed between +/+ and −/− aortic smooth muscle in response to $K^+$ (72.7 mM) stimulation (data not shown). Tension redevelopment following a step shortening as a function of time during contraction was measured, from which qualitative estimates of crossbridge kinetics can be inferred (1, 30). Figures 3A and 3B illustrate the force redevelopment ($F/F_0$) at 1 min and 5 min after K+ stimulation, where $F_0$ is a fall in force by a step shortening and is nearly the same as the isometric force just before a release and F is force redevelopment after a release. In both +/+ and −/− muscles, the rate of force redevelopment was faster in the release at 1 min than at 5 min after the start of the stimulation. In the early time course of the response in the release at 1 min, there was no significant difference between +/+ and −/− muscles. As shown in Fig. 3B, however, in the release at 5 min after the start of the stimulation, force redevelopment of −/− muscle was significantly increased at any time point as compared with +/+ muscle. In both early and late phase of contraction, there were no differences in the amounts of phosphorylated myosin regulatory light chain and in intracellular $Ca^{2+}$ concentrations at any time point after $K^+$ stimulation between +/+ and −/− muscles (Fig. 3C), indicating that the level of activation of contractile apparatus was similar in +/+ and −/− muscles.

*Contraction and shortening velocity in vas deferens smooth muscle.* In the contraction of vas deferens stimulated by high $K^+$ (140 mM), the peak tension of phasic contraction was significantly reduced in −/− muscle, while there was no difference in tonic force following the phasic one between +/+ and −/− muscles (Fig. 4). Relation between force and velocity were determined during phasic contraction by applying isotonic release at the peak of force. The velocity was measured at 0.2 s after the release under various relative loads and force-velocity curve was drawn. The shortening velocity at zero external load ($V_{max}$) as an estimate of the crossbridge cycling rate and $a/P_0$, where $a$ is a constant in Hill equation and

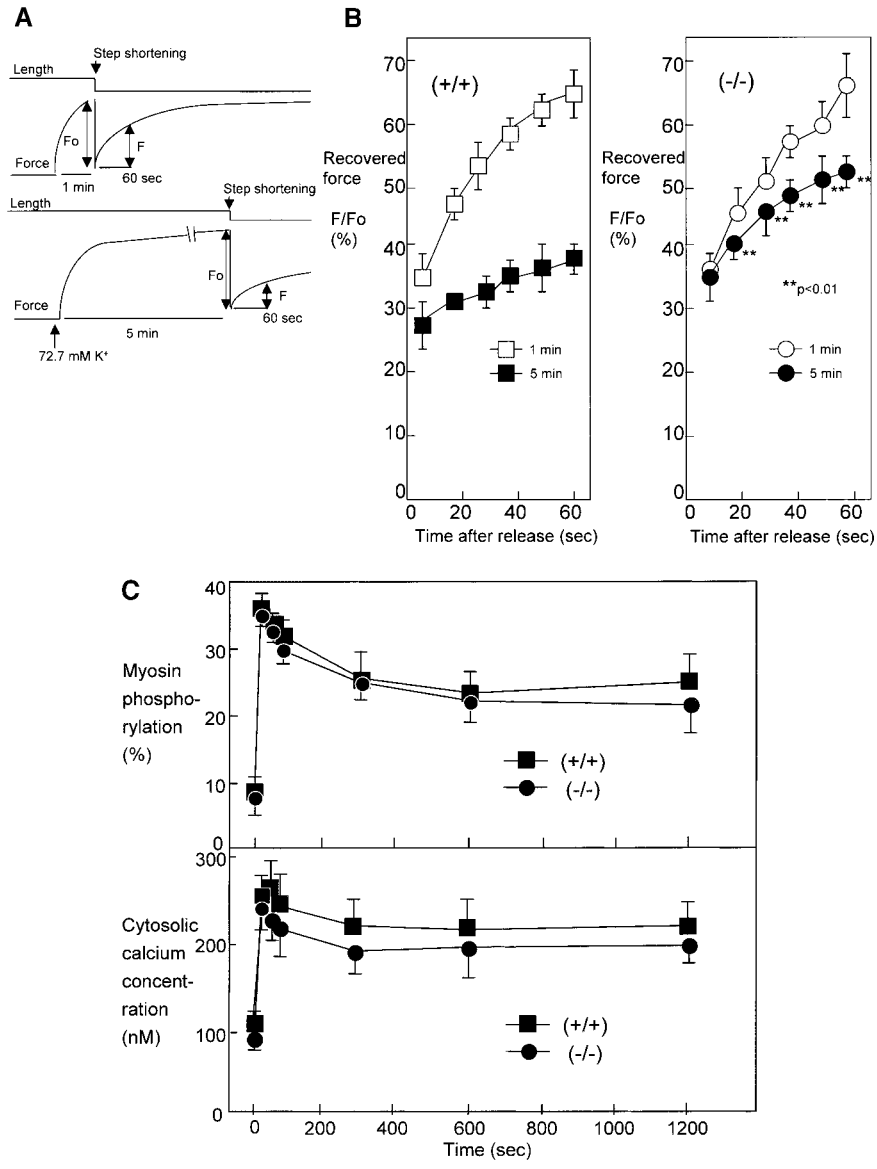


**FIG. 3.** (A) Schematic drawing of a step shortening in length and the resultant force changes. Step shortening (ca. 1% of initial length) was imposed at 1 and 5 min after $K^+$ (72.7 mM) stimulation. $F_0$ is a fall in force by a step and is nearly same as isometric force just before a step and $F$ is a force redeveloped. (B) Force redevelopment ($F/F_0$) after a step shortening in +/+ and −/− aortic smooth muscles ($n = 6$ per group). Asterisks in −/− muscle mean the significant difference from +/+ muscle ($P < 0.01$). The data are means ± SE from three independent experiments. (C) Fractional phosphorylation of myosin regulatory light chain and intracellular $Ca^{2+}$ concentrations in +/+ and −/− aortic smooth muscles as a function of time after $K^+$ depolarization. The experiments were repeated twice, and the data are means ± SE ($n = 5$ per group for phosphorylation and $n = 9$ per group for $Ca^{2+}$).

$P_0$ is isometric force, were obtained from the linear form of Hill equation. The example of force–velocity curves illustrated in Fig. 5B and the comparison of the average of four samples (Fig. 5C) show that there were marked increases in shortening velocity in −/− as compared with +/+ muscle. An example of the difference in the shortening velocity under the same relative load (0.6 $P_0$) in −/− and +/+ muscles is shown in Fig. 5A. The parameter of $a/P_0$ is a measure of the concavity of the curve and is also a measure of detachment rate of actomyosin according to Simmons and Jewell's discussion (31) (Fig. 5C). These data clearly indicate that crossbridge cycling rate increases in calponin deficiency by the change in the kinetics of the interaction between actin and myosin.

*Length–force relationships.* Length–force relationship was recorded in the range of 1.0–1.4 × resting muscle length ($L_0$). In both +/+ and −/− muscles from aorta and vas deferens, stretching the muscles to approximately 1.2 $L_0$ resulted in development of the maximum active tension (data not shown).

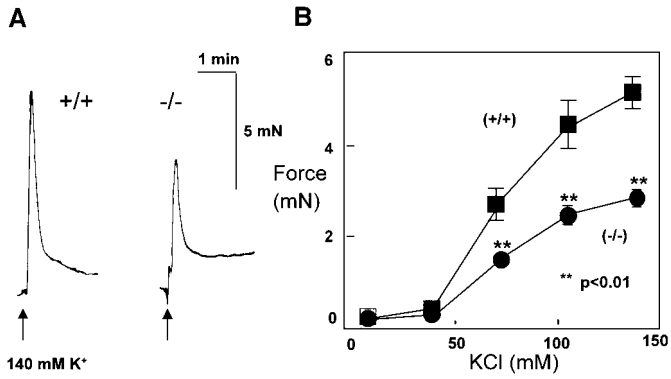


**FIG. 4.** (A) Effects of calponin deficiency on isometric force in intact muscle strips (5 mm in length and 0.5 mm in width) prepared from vas deferens. Contraction was initiated by 140 mM $K^+$. (B) $K^+$ concentration-dependent force production in transient phase of contraction of +/+ and −/− vas deferens smooth muscles. The experiments were repeated twice, and the data are means ± SE ($n$ = 5 per group).

## DISCUSSION

We generated mice lacking smooth muscle calponin and examined the mechanical properties of tonic and phasic smooth muscles. During the course of tonic contraction of aortic smooth muscle, force redevelopment after step shortening was significantly increased in −/− muscle as compared with +/+ muscle with similar levels of activation estimated by phosphorylation levels of myosin light chain and intracellular $Ca^{2+}$ concentrations. We also showed that there was a large increase in the $V_{max}$, the maximum shortening velocity when no external load exists, in −/− muscle from vas deferens. These observations are consistent with the notion that calponin is involved in a myosin phosphorylation-independent mechanism for slowing shortening velocity (13–15, 19, 20) in both tonic and phasic smooth muscles but not consistent with a hypothesis that calponin plays a role in maintaining a relaxed state and inhibiting the formation of latch bridges (20).

If calponin is an integral component of the latch state (19) and calponin inhibition occurs primarily on unphosphorylated myosin *in vivo* (20), effects of calponin deficiency on the crossbridge cycling rates will become more apparent in the sustained phase of contraction. We therefore compared force redevelopment following isometric step shortening at 1 and 5 min after $K^+$ depolarization in aortic smooth muscle. The present results showed that an increase in the recovered force and thus an increase in the crossbridge cycling rate indeed occurred only at 5 min after stimulation during a maintained contraction, in which fractional phosphorylation of myosin light chain was reduced. Murphy and colleagues have suggested that there is a transformation of cycling crossbridges to latch-bridges during the course of the contraction leading to the fall in shortening velocity (2, 5). Calponin may be a candidate determinant of the interchange between cycling and non-cycling crossbridge species during contraction.

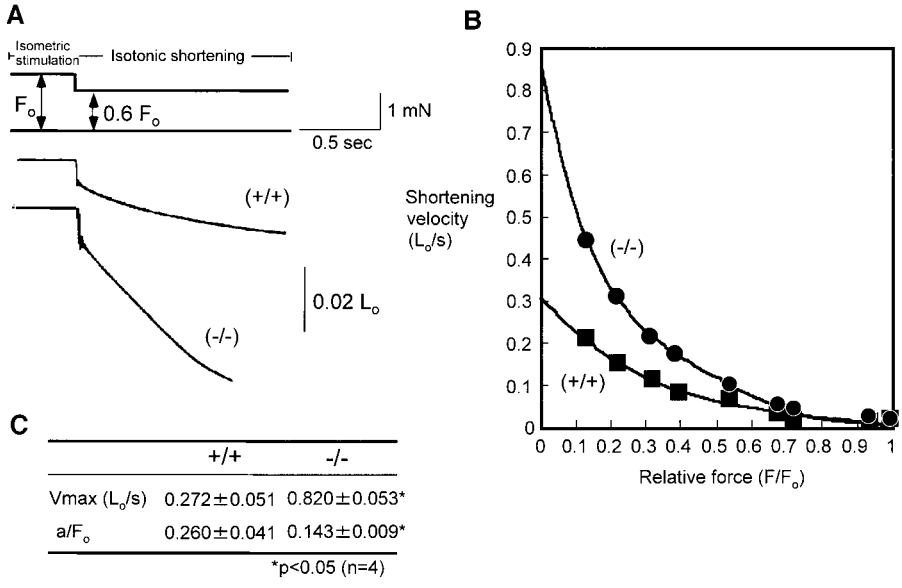


**FIG. 5.** Comparison of isotonic shortening between +/+ and −/− vas deferens smooth muscles stimulated by 140 mM $K^+$. (A) The shortenings following a quick isotonic release to the same relative load of 0.6 $P_0$ ($P_0$: isometric force just before a release). (B) An example of complete force–velocity curves constructed from isotonic quick release under various relative loads. Velocities were measured from the slope of the length record at 0.2 s after a release. The curves are fitted by the hyperbolic Hill equation. (C) $V_{max}$, the maximum shortening velocities at zero external load and $a/P_0$ ($a$, constant in Hill equation). The data are means and ± SE ($n$ = 4 per group). Note that the shortening velocities are clearly faster in A, B, and C in −/−. Read text for details.

Our results demonstrating that the maximum velocity of shortening at zero external load is more than two times faster in early phase of −/− muscle contraction from vas deferens may suggest that calponin inhibits phosphorylated myosin *in vivo*. It has been reported that exogenous calponin inhibits phosphorylated myosin ATPase activity *in vitro* (19, 32, 33) and shortening velocity of skinned muscle fibers *in vivo* (14, 15) when myosin light chain was thiophosphorylated. Alternatively, calponin may inhibit a rapidly accumulating dephosphorylated myosin during a transient phase of contraction of the phasic muscle.

Based on the biochemical studies, Haeberle has proposed that calponin inhibits the rate of dissociation of high affinity crossbridges from actin, and consequently, increases the force production by unphosphorylated myosin and decreases unloaded velocity (19, 34). If this model is correct, calponin deficiency should facilitate the dissociation of high affinity crossbridges from actin, and consequently, decreases force production with increased unloaded velocity. The results presented here is consistent with this model. As compared with +/+ muscle, −/− muscle from vas deferens displayed decreased force production with increased unloaded shortening velocity. A fundamental question concerning physiological role of calponin in regulating crossbridge cycling rate is a mechanism for overriding the calponin inhibition during activation. Several mechanisms are implicated, including $Ca^{2+}$-stimulated phosphorylation (32), interaction with protein kinase C (35, 36), phosphorylation by Rho-kinase (37), $Ca^{2+}$/caltropin (38), and a conformational change of thin filament induced by the attachment of strongly bound phosphorylated crossbridges (19, 20, 34, 39).

In conclusion, the knockout mice strategy provided a powerful tool for analyzing physiological role of calponin in intact contracting smooth muscle cells. Our results, together with the future analysis of the energetic behavior of −/− muscle will provide a possible framework for understanding the molecular mechanisms responsible for regulated crossbridge cycling rates observed as variable shortening velocities and energy consumption in mammalian smooth muscles.

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