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Source: Zoological Science, 19(2) : 167-174

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.19.167>

Altered Mechanical Properties in Smooth Muscle of Mice with a Mutated Calponin Locus

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ABSTRACT—The mechanical properties of smooth muscles in aorta and vas deferens were studied in mice with a mutated basic calponin locus to learn the physiological function of calponin. The intact smooth muscles were stimulated with high KCl and the force development was compared between calponin deficient (knockout, KO) mice and wild type (WT) ones. The isometric force induced by various concentrations of high KCl was lower in KO than in WT both in aorta and in vas deferens. The length-force relations were compared between KO and WT. The active isometric force in KO was significantly lower at most muscle lengths examined than in WT without the change in resting force both in aorta and in vas deferens. In vas deferens, the rate of force development after quick release in length at the peak force was significantly faster in KO than in WT. The above results show that the force development is lower and the rate of cross-bridge cycle is faster in KO mice than in WT ones, suggesting that calponin plays basic roles in the control of the contraction of smooth muscle.

Key words: calponin, latch mechanism, smooth muscle, aorta, vas deferens

INTRODUCTION

Smooth muscle shows characteristic contractions different from those in skeletal muscle, which shows very fast shortening velocity and the cross-bridge cycling and these parameters do not change basically (Sugi and Tsuchiya, 1998). Smooth muscle shows maintained force and slower shortening and they are regulated by the intra- and extra-cellular factors. Catch contraction in mollusks and latch contraction in vertebrates are the examples of the regulation in smooth muscle (Sugi and Tsuchiya, 1979; Ishii *et al.*, 1997; Dillon *et al.*, 1981). It is demonstrated that the maximum shortening velocity and myosin light chain phosphorylation have maximal values early in the contraction and then decrease, but tonic force remains in the later phase of contraction (Dillon *et al.*, 1981). Now some lines of evidence are available to indicate that reversible Ca/calmodulin-dependent phosphorylation of myosin regulatory light chain primarily regulates the rate of ATP hydrolysis of smooth muscle myosin (Hartshorne, 1987; Somlyo and Somlyo, 2000). It has been shown later, however, that the shortening velocity,

i.e. cross-bridge cycling, and myosin ATPase activity can change independently of myosin light chain phosphorylation (Hori *et al.*, 1992; Sato *et al.*, 1992).

Basic calponin is a smooth muscle-specific and actin-binding protein isolated from bovine aorta and chicken gizzard (Takahashi *et al.*, 1986; Takahashi *et al.*, 1988) and the roles in physiological function have been discussed (Ito *et al.*, 1994; Horowitz *et al.*, 1996; Uyama *et al.*, 1996; Winder and Walsh, 1996). Some lines of evidence have been accumulated to indicate the *in vitro* biochemical properties of calponin (Haerberle *et al.*, 1994; Hodgkinson *et al.*, 1997; Malmqvist *et al.*, 1997). To elucidate the function of calponin physiologically, mice with a mutated basic calponin locus were generated and the mechanical properties of smooth muscle in intact tissue were compared between calponin knockout (KO) mice and wild type (WT) one (Takahashi *et al.*, 1998; Matthew *et al.*, 2000; Takahashi *et al.*, 2000). The present experiment is a further detailed study of the mechanical properties, especially about force development in the smooth muscles of aorta and vas deferens in intact tissues in calponin KO and WT mice.

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MATERIALS AND METHODS

A targeted mutation was induced in CCE ES cells and F2 mice homozygous for the mutant basic calponin alleles or the wild-type allele were generated after outcross to B57BL/6 mice as previously described (Yoshikawa *et al.* 1998). Aorta and vas deferens were isolated from 3~4-week-old male mice after killing by neck disarticulation. The tissue was cut into a ring preparation (0.8–1.2 mm in width) in aorta, which was mounted directly on the two arms from the force transducer and the servo-motor, or cut into a longitudinal strip in vas deferens (5–7 mm in length, 0.4–1.3 mm in width), to which aluminum clips were attached at both ends (Fig. 1). When active and resting forces were compared, they were normalized by the width of the preparation and expressed as the force per 1.0 mm and 0.5 mm in aorta and vas deferens, respectively. This way of normalization of force depended on the histological observation shown in the previous paper (Takahashi *et al.*, 2000). The composition of the Ringer solution (mM) is : NaCl, 136.9; KCl, 5.4; CaCl₂, 1.5; MgCl₂, 1.0; NaHCO₃, 23.8; EDTA (ethylenediamine-N,N,N',N'-tetraacetic acid) 0.01; glucose, 5.5. The solutions containing 72.7 or 140 mM KCl were used for the stimulation in the most experiments of aorta and vas deferens, respectively and were made by substituting NaCl with equimolar KCl in the normal Ringer. These solutions were saturated with 95% O₂ and 5% CO₂ mixture at 37°C and pH 7.4 in the course of all experiments. The preparations were equilibrated for 45–60 min in a 10 ml bath until the contractile response to high KCl solution became stable. In the measurements of isometric force in aorta, the length-force relation was first measured at several lengths and the optimal length for force development was found or the muscle length was adjusted to optimum length according to the results of length-force relationships. In the measurements of isometric force in vas deferens, the length of a preparation was adjusted to the length with only slight resting force. Force change was measured by a transducer (AE801, Sensoron)

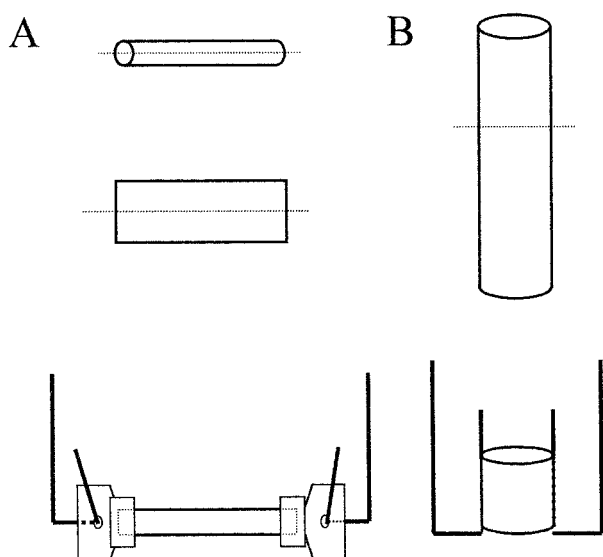


Fig. 1. Schematic drawing of the method to attach a preparation to a force transducer. **A** Tubular vas deferens tissue was cut open and aluminum connectors were attached to a strip preparation (5–7 mm in length, 0.4–1.3 mm in width) at both ends. A preparation was set to the arms from a force transducer and a servo-motor. **B** Tubular aorta tissue was cut into a ring preparation (0.8–1.2 mm in width) and it was set to the arms from a force transducer and a servo-motor.

with a compliance of 0.1 mm/N and a resonant frequency of about 5 kHz or by the feed back signal from the servo-motor which could generate force and length steps (Dual Mode Servo 300, Cambridge Technology; Sugi and Tsuchiya, 1988). The quick length steps were applied by the servo-motor within 2 ms. The force and length changes were recorded with a digital oscilloscope (AR1200, Yokokawa or type 310, Nicolet). The force redevelopment after a length step in vas deferens was measured by imposing about 6.0% shortening steps at the top of the isometric force and the rate of force rise after release was expressed as P_1/P_0 , where P_0 is the isometric force and P_1 is the extrapolated height of the slope immediately after the release to 1 s after a release as shown in Fig. 7. In the measurement of length-force relation, the preparation was stimulated by high KCl in vas deferens and aorta 1 min and 5 min after the change of the length, respectively. The active force was calculated by the subtraction of the resting force from the total force. The force was measured at the peak in vas deferens and at 15 min after the start of the stimulation in aorta. The stimulation was made every 20 and 50–60 min in vas deferens and aorta, respectively.

All data are expressed as means \pm S.D. Student's t test for independent samples was used as a post hoc test of significance, with significance set at $p < 0.05$.

RESULTS

Time course of force development

vas deferens In both KO and WT mice, vas deferens preparations developed force immediately after the stimulation by various concentrations of high KCl and the activated force reached the peak at 10–15 s and then fell to the steady level of 20–25% of the peak height for 30–40 s at 140 mM KCl as shown in Fig. 2. The former quick response, so-called phasic response, and the later maintained one, so-called tonic response were observed in KO and WT at all high KCl concentrations and at all lengths of the preparations. Therefore, the distinct differences in the time course of the force development were not observed between KO and WT mice.

The resting force developed quickly after the stretch of a preparation and decayed exponentially, first quickly and then slowly in the later phase in both KO and WT mice as in other smooth muscles (Fig. 2). The magnitude of the resting force was different especially in the longer length both in WT and KO mice as seen in Fig. 6 and the peak resting force immediately after the stretch largely depended on the stretch velocity both in WT and KO. The clear differences in the time course of the resting force after stretch were not observed between KO and WT.

aorta In both KO and WT mice, aorta preparations developed force gradually after the stimulation and the activated force reached the plateau after 15 min, which was maintained in the later phase at all high KCl concentrations and at all lengths of the preparations examined (Fig. 3). The detailed analysis of the rising phase of force by the stimulation showed that the rate of rise immediately after the stimulation in KO is a little faster than in WT (Fig. 4A). After the end of the stimulation, force started to decrease and it took about 15 min to return the exact resting force level. The fall-

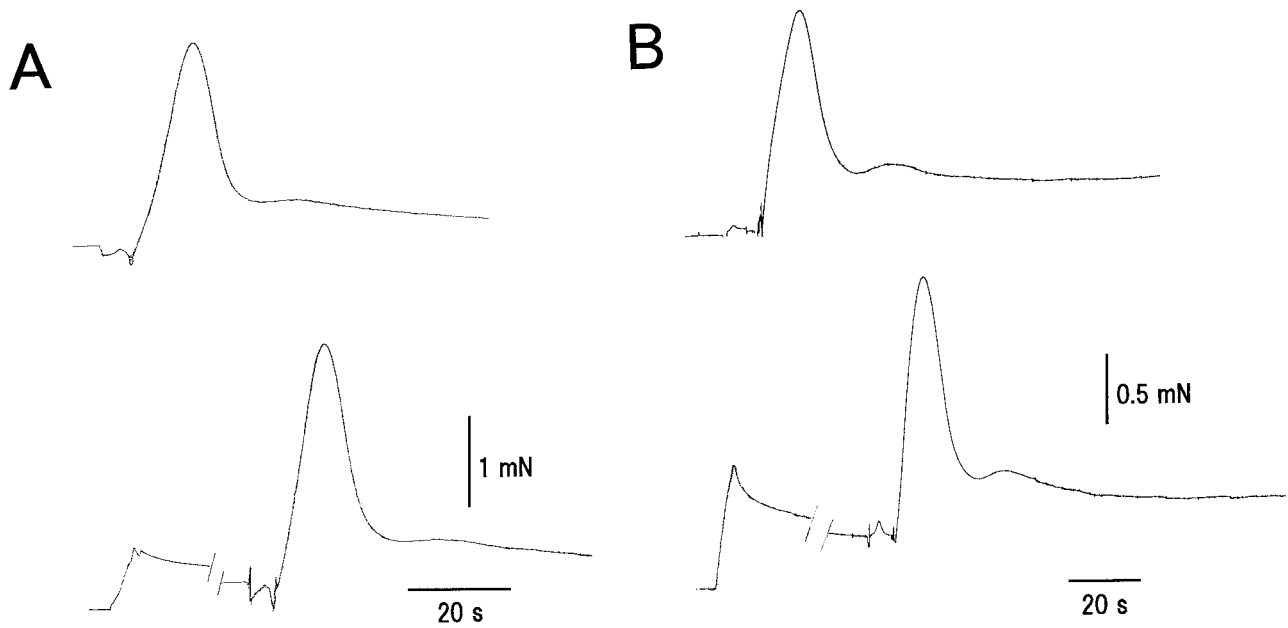


Fig. 2. Comparison of the force development by 140 mM KCl in vas deferens between wild type (WT) mice and calponin knockout (KO) ones. **A** Force development in vas deferens in WT at 1.0 lo (lo; resting muscle length)(upper trace) and at 1.20 lo (lower trace). **B** Force development in KO at 1.0 lo (upper trace) and at 1.24 lo (lower trace). Note that the active force height is lower in KO than in WT in the same width of preparations.

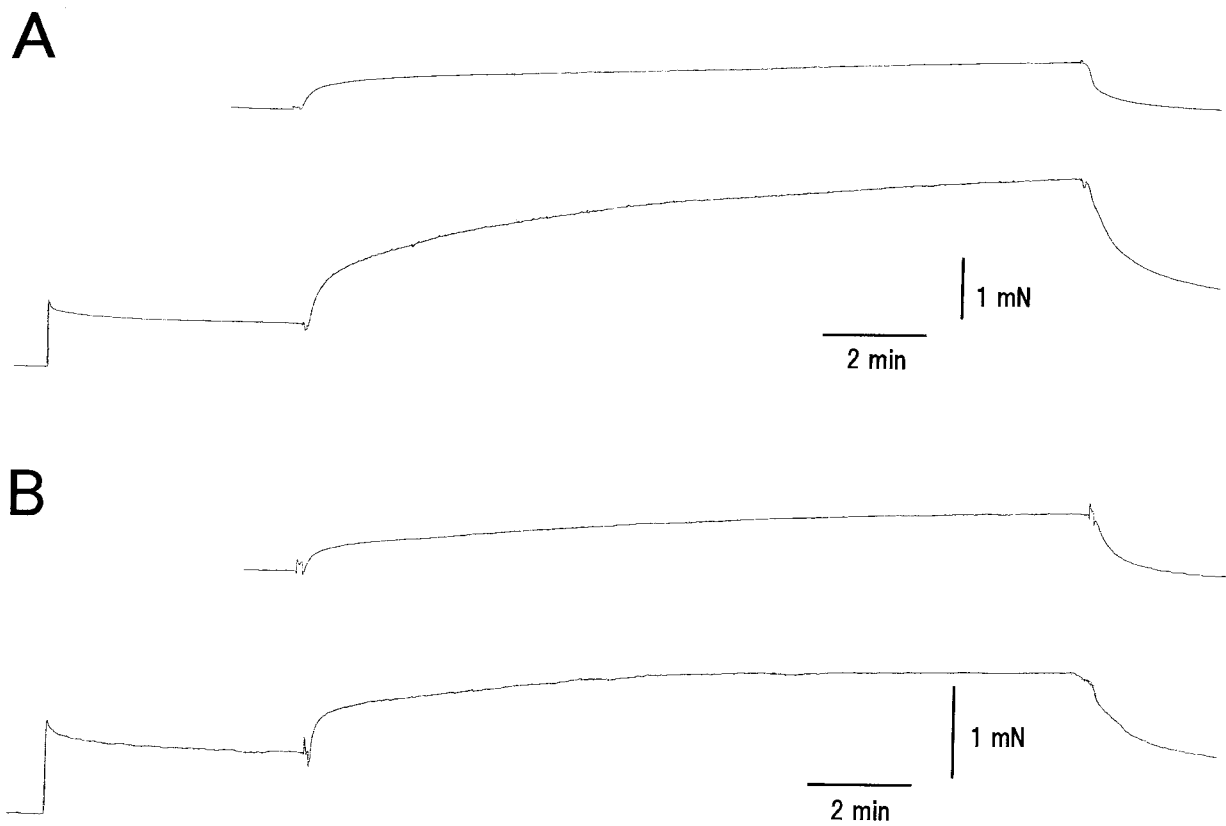


Fig. 3. Comparison of the force development by 72.7 mM KCl in aorta between WT and KO of nearly same width of preparations. **A** Force development in WT at 1.0 lo (lo; resting muscle length)(upper trace) and at 1.20 lo (lower trace). **B** Force development in KO at 1.0 lo (upper trace) and at 1.22 lo (lower trace). The time courses of the development and the decline of force were analyzed in Fig. 4.

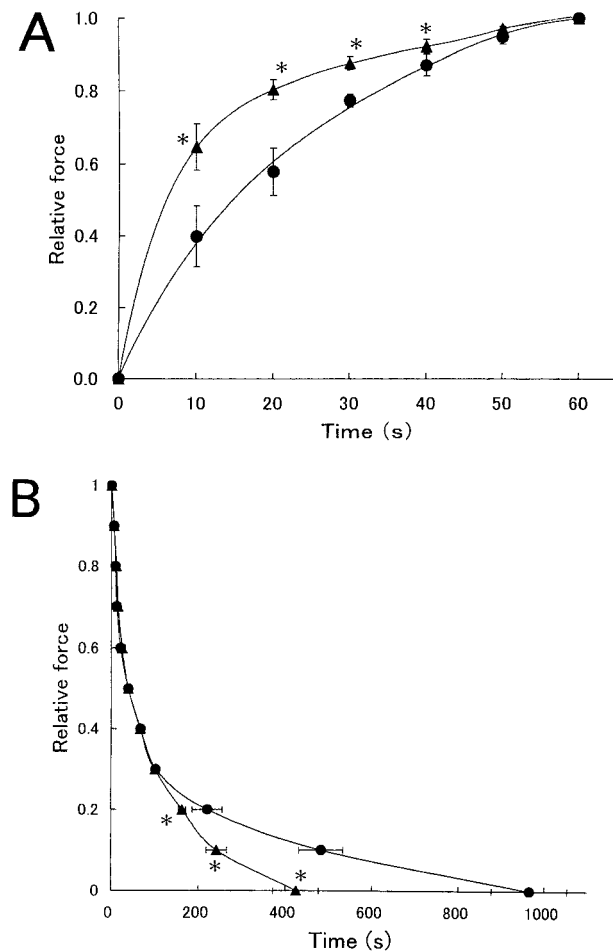


Fig. 4. The comparison of the time courses of the force development in aorta after the application of 72.7 mM KCl (A) and of the force decline after the cessation of the stimulation (B) between WT (●) and KO (▲). The force is expressed as the relative value to the one at 60 s after the application of high KCl in A and to the one just before the cessation of the stimulation in B. Data points and bars show the means and the standard deviations ($n=4$). Data points with asterisks mean that the differences between WT and KO are statistically significant ($p<0.01$). Note that the time course of the rise and the fall of force is faster in KO than in WT.

ing phase of force after the end of stimulation is significantly faster in KO than in WT at the later phase of relaxation (Fig. 4B). The resting force rose rapidly after the stretch of the preparation and decayed first quickly and then slowly in later phase (Fig. 3). The magnitude of the resting force was different especially in the longer length both in WT and KO mice as seen in Fig. 7 and the peak resting force immediately after the stretch largely depended on the stretch velocity both in WT and KO. The clear differences in the time course of the resting force after stretch were not observed between KO and WT.

Active isometric force

vas deferens The amplitude of the activated phasic force in *vas deferens* was measured at 40, 70, 105, and 140 mM KCl in KO and WT (Fig. 5A). In both types of mice, the force

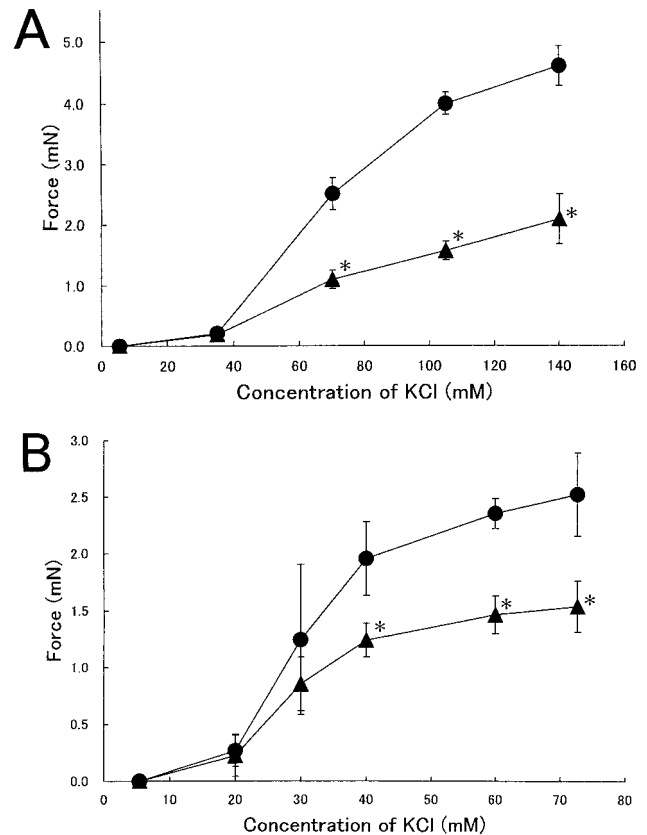


Fig. 5. Comparison of active force developments by various concentrations of KCl in *vas deferens* (A) and *aorta* (B) between WT (●) and KO (▲). Data points and bars show the means and the standard deviations ($n=7-8$ in *vas deferens* and $4-7$ in *aorta*). Forces are normalized by the width of preparations and expressed as $mN/0.5\text{mm}$ and $mN/1.0\text{mm}$ in *vas deferens* and *aorta*, respectively. Data points with asterisks mean that the differences between WT and KO are statistically significant ($p<0.01$). Note that the forces in KO are clearly lower than in WT.

depended on the concentration of KCl and was about 40–50% and 70–85% of the maximum force at 70 and 105 mM KCl, respectively. When the relative force was compared between KO and WT after the normalization by the width of the preparation ($mN/0.5\text{mm}$), the difference was remarkable and statistically significant, the amplitude in calponin KO being about half of that in WT.

aorta The amplitude of the activated force, normalized by the width of preparations, in *aorta* was measured at 20, 30, 40, 60 and 72.7 mM KCl and was calculated by the subtraction of the resting force from the total force both in KO and in WT (Fig. 5B). In both types of mice, the force depended on the concentration of KCl. The force increased with the concentration and it was about 50, 80 and 95% of the maximum force at 30, 40 and 60 mM KCl, respectively. When the relative force was compared between KO and WT after the normalization by the width of the preparation ($mN/1.0\text{mm}$), the difference was remarkable and statistically significant in *aorta* as well, the amplitude in calponin KO mice being about 60% of that in WT.

Length-force relations

vas deferens The length-force relations were examined in *vas deferens* stimulated by 140 mM KCl at the muscle length between 0.6–1.5 lo (lo; just the length with a very small resting force) in both types of mice. The mean forces in four preparations are shown in Fig. 6A, B, in which the force is normalized by the width of preparations. In both KO and WT mice, the resting force increased steeply above 1.2 lo with the increase in the length. The resting force exceeded the active one above 1.3–1.4 lo. The active force was scarcely observed at 0.6 lo and increased with the increase in the muscle length and the maximum force was

produced at around 1.2 lo in both types of mice. The most noticeable difference between KO and WT was observed in the active force, the force in KO being lower than half of that in WT at optimum length. The difference in active force was statistically significant as shown in Fig. 6A, B. The height of the resting force, however, is similar in both types, namely the difference was not statistically significant (Fig. 6A, B). *aorta* The length-force relations were examined in *aorta* stimulated by 72.7 mM KCl at the muscle length between 0.8–1.5 lo in both types of mice. The mean forces ($n=4-8$) are shown in Fig. 7A, B, in which the force is normalized by the width of preparations. In both types of mice, a small rest-

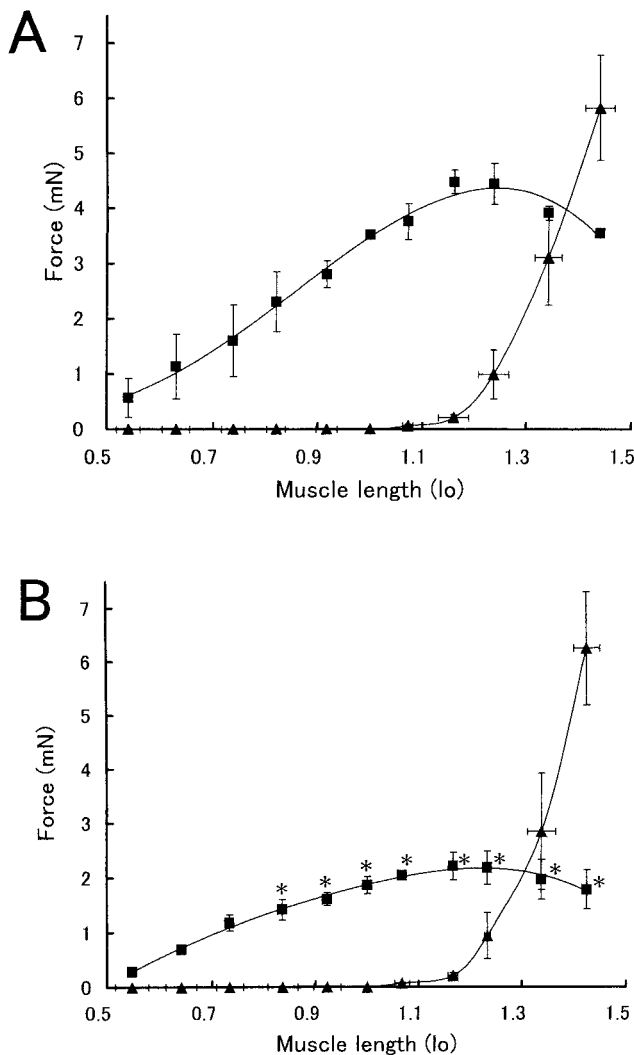


Fig. 6. Comparison of the length-force relations in *vas deferens* between WT (A) and KO (B). Active (■) force induced by 140 mM KCl and resting one (▲) are compared after the normalization of force by the width of the preparations, expressed as mN/0.5 mm. Data points and bars show the means and the standard deviations ($n=4$). The horizontal error bars in active force are the same as ones in resting force and omitted. Data points with asterisks mean that the differences between WT and KO are statistically significant ($p<0.01$). Note that the active force is clearly lower in KO than in WT, while there is no significant difference in the resting force between both types.

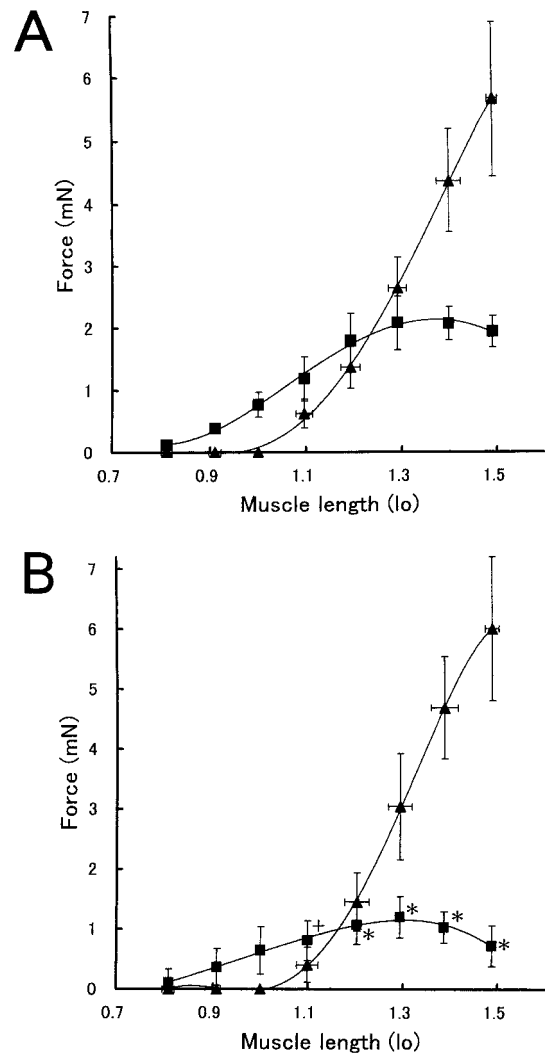


Fig. 7. Comparison of the length-force relations in *aorta* between WT (A) and KO (B). Active force induced by 72.7 mM KCl (■) and resting one (▲) are compared after the normalization of force by the width of the preparations, expressed as mN/1.0 mm. Data points and bars show the means and the standard deviations ($n=4-8$). The horizontal error bars in active force are the same as ones in resting force and omitted. Data points with asterisks mean that the differences between WT and KO are statistically significant ($p<0.01$). Note that the active force is clearly lower in KO than in WT, while there is no significant difference in the resting force between both types.

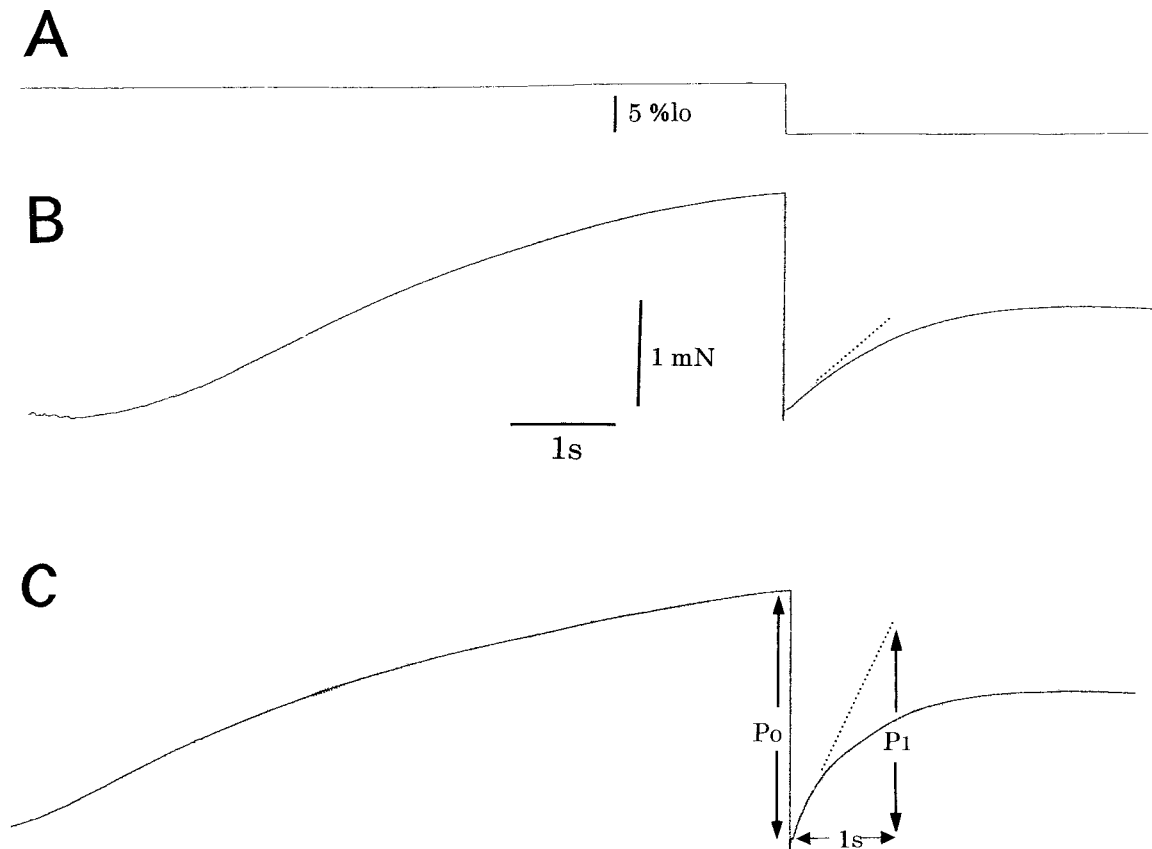


Fig. 8. The records for the comparison of the rate of force redevelopment after a quick shortening step of 6% lo (**A**) in WT (**B**) and KO (**C**) during the contraction induced by 140 mM KCl. Read Methods about P_0 , P_1 and the procedure for the calculation of the rate of rise after a shortening step.

ing force was observed at 1.1 lo and it increased with the increase of the length. The active force was scarcely observed at 0.8 lo and increased with the increase in the muscle length, the maximum force being produced at around 1.3 lo in both types of mice. The amplitude of active force is markedly lower in KO than in WT at 1.1–1.5 lo and the difference is statistically significant. In contrast, the difference in the resting force between both types is not statistically significant.

Rate of rise after quick release

The preparation of vas deferens was quickly released by a length step at the maximum force induced by 140 mM KCl and the rate of force rise was measured (Fig. 8). The force quickly dropped in accordance with a quick length step and it started to rise from zero force level. The amplitude of a step was adjusted so that the bottom force after the release was just at zero level (Fig. 8A). If the release distance exceed a certain limit, the lowest force stayed for a while at 0 level. The rate of rise was different in the different points of time after the stimulation and it was slower at the beginning of the stimulation and also after the peak force, therefore, the rate of rise was always measured at the peak force. The typical records are shown in Fig. 8. In WT (Fig.

8B), force started to rise relatively slowly from the bottom after a step and rose up to 40–50% of the peak force. This incomplete recovery of force might be caused by the decrease in the activation by high KCl. The force in KO rose from the bottom faster than in WT (Fig. 8C). The essential difference between two types of mice was only the rate of rise and no other difference was found in force change. The rate of rise was measured as mentioned in the methods and compared between WT and KO. The results (from 5 sam-

Table 1. Comparison of the rates of force redevelopment after a shortening step in vas deferens between wild-type (WT) and calponin knockout (KO) during the contraction induced by 140 mM KCl. The difference in the mean of force is statistically significant ($p < 0.001$). See text for details.

rate of rise (P_1/P_0)	
WT	KO
0.31	0.70
0.41	0.74
0.54	0.86
0.33	0.84
0.23	0.82
0.36 ± 0.12	0.79 ± 0.07

ples in both types of mice are shown in Table 1. The means are 0.36 and 0.79 in WT and KO, respectively and the difference was statistically significant ($p < 0.001$).

DISCUSSION

The contraction of mammalian smooth muscle is regulated primarily by the phosphorylation and dephosphorylation of myosin (Dillon *et al.*, 1981; Walsh *et al.*, 1982; Hartshorne, 1987). The binding of Ca and calmodulin (CaM) forms the Ca-CaM complex, which interacts with the enzyme, myosin light-chain kinase (MLCK) and converts kinase from an inactive to an active state. This phosphorylation reaction triggers the cycling of myosin cross-bridges along actin filaments and develops contractile force. The lines of evidence have been accumulated to support a primary role played by myosin phosphorylation and dephosphorylation in the regulation of mammalian smooth muscle contraction. But a lot of physiological and pharmacological studies have suggested the existence of additional secondary mechanisms, Ca-dependent and Ca-independent, which modulate the contractile state of a mammalian smooth muscle (Hori *et al.*, 1992; Sato *et al.*, 1992).

In the present paper, we demonstrated that the some mechanical properties in calponin knockout mice are different from those in wild type ones. The most remarkable point is the fact that the isometric force in KO elicited by high KCl is clearly lower than in WT in length-force relations (Figs. 6 and 7). In our previous paper (Takahashi *et al.*, 2000) also, the decrease in isometric force was suggested. The isometric force, however, primarily depends on the muscle length and the exact proof of the isometric force necessitates the measurements of length-force relationships. In Figs. 6 and 7, it is definitely proved that the active isometric force is lower in KO than in WT and this difference is statistically significant. On the other hand, there is no statistical difference in the resting force between two types of mice in vas deferens and aorta. This fact suggests that the tissues causing the resting force are not modified by the genetic deficiency of calponin. We studied the histology of smooth muscle tissues of vas deferens and aorta in KO and WT in the previous paper (Takahashi *et al.*, 2000) by light- and electron-microscopy. There appeared no obvious differences between KO and WT in wall thickness, the number of cell layers, cell shape or size, and extracellular matrix. The only difference was that the histochemical study did not detect calponin in KO mice. These histological observations are consistent with the present measurements of resting force.

The lower active force generation in KO groups was also confirmed by the results shown in Fig. 5, in which the concentration of KCl for stimulation was changed. The first step for activation by KCl is the depolarization of membrane, which activates Ca influx or Ca release from intracellular store-site. The results in Fig. 5 suggest that the activation process through membrane depolarization was not altered by calponin deficiency, though membrane potential was not

measured in the present experiment. In our previous experiment (Takahashi *et al.*, 2000), we measured the intracellular Ca concentration and proved that the changes in the intracellular Ca concentration with time in the activation by KCl in KO was not different from those in WT. These observations may suggest that the genetic calponin deficiency does not influence the excitation-contraction coupling, though further experiments would be required for the exact proof.

In our previous paper (Takahashi *et al.*, 2000), it was shown that the maximum isotonic shortening velocity of the smooth muscle in vas deferens in calponin KO mice was faster than in WT. In the present experiment, we showed that the rate of rise after a quick length step, which was also a measure of the cycling of cross-bridges (Carlson and Wilkie, 1974), was faster in KO by more than twice than in WT (Fig. 8) and this difference was statistically significant. These results demonstrate that cross-bridge cycling in calponin KO is faster both in the isometric and in isotonic condition than in WT.

It was observed in aorta that the time courses of force development by KCl and of force decrement after the end of the stimulation were faster in KO than in WT (Fig. 4). One possible interpretation of these facts is that the cross-bridge cycling is faster in KO. The processes, however, of the activation and deactivation by high KCl are very complex and some other explanations are possible. As studied previously (Takahashi *et al.*, 2000), the thickness and the structure of the tissue in KO is similar to those in WT and there may be no difference in the time for diffusion of the solution, therefore, the cause may exist in intracellular mechanism, e.g. the change in Ca concentration, detachment and attachment of calponin from and to actin, if any, and phosphorylation and dephosphorylation of myosin light chain. Among these, we measured the changes in intracellular Ca concentration and in the phosphorylation in the previous paper (Takahashi *et al.*, 2000) but the sampling rate of measurement was not fast enough to explain the present results.

It is interesting to infer the mechanism of the cross-bridge cycling from the present results that the calponin deficiency elicits the decrease in the force production and the increase in the rate of cross-bridge cycling. According to the hypothesis of muscle contraction (Huxley, 1957; Simmons and Jewell, 1973), the kinetic interaction between actin and myosin is determined by the rate constants of attachment (f) and detachment (g) between them, namely the isometric force and the maximum sliding velocity is determined by $f/(f+g)$ and $f+g$, respectively. If g is assumed to increase in calponin deficiency, the isometric force decreases and the velocity increases. According to the hypothesis of the cycle of ATP-hydrolysis (Taylor, 1972), the rate limiting step in the cycle is the release of Mg-ADP from actin-myosin complex. Using an *in vitro* motility assay, Haeberle (1994) demonstrated that calponin increased the force exerted by myosin and stationary actin filaments by 3–4 fold and decreased sliding velocity of actin filament on myosin. Based on these results, Haeberle suggested that calponin inhibits the rate of

dissociation of the high-affinity actomyosin complex and concluded that calponin may be an integral component of the latch-state in mammalian smooth muscle. Our present results together with the previous one (Takahashi *et al.*, 2000) using knockout mice strategy are quite similar to those *in vitro* motility assay above-mentioned. The exact mechanism by which calponin modulates the interaction between actin and myosin is not clear but our recent unpublished data and the works *in vitro* by others demonstrate that calponin causes a marked inhibition of the actin activated myosin ATPase (Winder and Walsh, 1990; Abe *et al.*, 1990). Further studies are required to elucidate the molecular basis of the inhibition of actin-activated myosin ATPase by calponin.

ACKNOWLEDGEMENTS

The authors would like to thank to Prof. Dr. H. Sugi in Teikyo University for the generous use of the electric apparatus and for some comments at the Annual Meeting on Muscle and Cell Motility Physiology and to Drs. H. Karaki, H. Ozaki and M. Hori for their invaluable suggestions during the course of the experiment.

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(Received August 21, 2001 / Accepted October 12, 2001)