The Smooth Muscle Cross-bridge Cycle Studied Using Sinusoidal Length Perturbations

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ABSTRACT The mechanical characteristics of smooth muscle can be broadly defined as either phasic, or fast contracting, and tonic, or slow contracting (Somlyo and Somlyo, 1968, *Pharmacol. Rev.* 20:197–272). To determine if differences in the cross-bridge cycle and/or distribution of the cross-bridge states could contribute to differences in the mechanical properties of smooth muscle, we determined force and stiffness as a function of frequency in Triton-permeabilized strips of rabbit portal vein (phasic) and aorta (tonic). Permeabilized muscle strips were mounted between a piezoelectric length driver and a piezoresistive force transducer. Muscle length was oscillated from 1 to 100 Hz, and the stiffness was determined as a function of frequency from the resulting force response. During calcium activation (pCa 4, 5 mM MgATP), force and stiffness increased to steady-state levels consistent with the attachment of actively cycling cross-bridges. In smooth muscle, because the cross-bridge states involved in force production have yet to be elucidated, the effects of elevation of inorganic phosphate (P_i) and MgADP on steady-state force and stiffness were examined. When portal vein strips were transferred from activating solution (pCa 4, 5 mM MgATP) to activating solution with 12 mM P_i, force and stiffness decreased proportionally, suggesting that cross-bridge attachment is associated with P_i release. For the aorta, elevating P_i decreased force more than stiffness, suggesting the existence of an attached, low-force actin-myosin-ADP- P_i state. When portal vein strips were transferred from activating solution (pCa 4, 5 mM MgATP) to activating solution with 5 mM MgADP, force remained relatively constant, while stiffness decreased ~50%. For the aorta, elevating MgADP decreased force and stiffness proportionally, suggesting for tonic smooth muscle that a significant portion of force production is associated with ADP release. These data suggest that in the portal vein, force is produced either concurrently with or after P_i release but before MgADP release, whereas in aorta, MgADP release is associated with a portion of the cross-bridge powerstroke. These differences in cross-bridge properties could contribute to the mechanical differences in properties of phasic and tonic smooth muscle.

INTRODUCTION

In the 1970s it was recognized that smooth muscle mechanics could be modeled using the same sliding filament/cross-bridge population paradigms as those for striated muscle (Huxley and Simmons, 1971). Although smooth muscle and skeletal muscle have similar basic mechanisms, there are crucial differences between the two that define their individual characteristics. Mechanically, smooth muscle is broadly categorized as either phasic or tonic (Somlyo and Somlyo, 1968). Phasic smooth muscle is characterized by relatively rapid rates of force activation and relaxation, high actomyosin ATPase activity, and a fast maximum velocity of muscle shortening (v_{max}). On the other hand, tonic smooth muscle has relatively slow rates of force activation and relaxation, a slow actomyosin ATPase, and a slow v_{max}. There are also differences in the splice variant expression of the myosin heavy chain (Kelley et al., 1993) and essential light chain (MLC_{17}) isoforms (Nabeshima et al., 1987) between phasic and tonic smooth muscle (Szymanski et al., 1998). These structural differences are thought to lead to differences in distribution and kinetics of the cross-bridge cycle (Morano et al., 1997) and the ADP affinity of the cross-bridge (Fuglsang et al., 1993; Nishiye et al., 1993; Khromov et al., 1995).

Traditionally, the quick release methods of force clamps (Hill, 1938) and length steps (Huxley and Simmons, 1971) have been used to investigate cross-bridge properties. In 1980, Kawai and Brandt developed a method that expanded this basic concept to the detection of shifts in the cross-bridge population, using separate, fixed-frequency sine wave measurements or fixed-frequency sinusoidal length perturbations. The fixed-frequency sinusoidal length perturbation protocol has a major advantage over traditional methods in that it is able to detect shifts in the cross-bridge population (Kawai and Zhao, 1993). However, this method requires multiple contraction and relaxation cycles, resulting in limited temporal resolution because the stiffness is determined separately at each frequency. To increase the accuracy, frequency, and temporal resolution of the fixed-frequency sinusoidal length perturbation protocol, a novel length perturbation protocol was recently developed in our laboratory that combined and linked linearly increasing multiplet sine waves or increasing multi-sine-wave length perturbation protocols (Shue and Brozovich, 1999). In tissue strip preparations, the increasing multi-sine-wave length perturbation protocol yields a linear frequency response of 1–100 Hz with a frequency resolution of 1 Hz and a temporal resolution of 14 s.

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Energy transduction in muscle is accomplished by a cyclic interaction between the globular head of myosin interacting with actin in the thin filaments. Relaxed muscle contains predominantly detached or weakly attached cross-bridges with bound products of ATP hydrolysis (refer to Fig. 1, *state a*). Upon activation, the cross-bridge attaches to the actin filament and tilts, to produce the sliding force between the thick and thin filaments. Force generation has been associated primarily with the release of P_i from the active site in smooth muscle (Fig. 1, *step 4*; Osterman and Arner, 1995; He et al., 1998). X-ray diffraction has predicted that the release of P_i from the active site results in local rearrangements of the protein structure near the active site, translating into a 5–20-nm movement of the light chain region at the head-rod junction (Rayment et al., 1993). However, in smooth muscle, force generation may also be associated with ADP release (Fig. 1, *step 6*; Whittaker et al., 1995; Gollub et al., 1996). The release of ADP from the S_1 nucleotide binding cleft results in the ~23° movement of the light chain binding helix, resulting in a ~35-Å movement of the last heavy chain residue. After ADP release (Fig. 1, *step 6*) in the presence of MgATP, there is rapid ATP binding (Fig. 1, *step 1*) that induces cross-bridge detachment (Fig. 1, *step 2*). Hydrolysis of ATP by the S_1 head reforms actin+myosin-ADP- P_i (A+M-ADP-P_i) (Fig. 1, *step 3*). Evidence suggests that two A-M-ADP-P_i states are present in skeletal muscle, and force is thought to occur during a rapid isomerization that immediately precedes P_i release (Geeves, 1991; Kawai and Halvorson, 1991; Dantzig et al., 1992). However, we have not included this state (A-M-ADP- P_i) in the scheme because there is no experimental evidence that it exists in smooth muscle (Fuglsang et al., 1993).

We chose to investigate the cross-bridge cycle in smooth muscle to determine if differences exist between tonic and phasic smooth muscle. In skinned smooth muscle, we reasoned that if an elevation of P_i changes the distribution of the cross-bridge states and increases the relative population of the A+M-ADP-P_i state (Fig. 1, *state a*), then an increase in MgADP should similarly result in an increase in the relative population of the A-M-ADP state(s) (Fig. 1, *states b* and *c*). Furthermore, if force generation is coincident with the release of P_i, elevation of P_i should result in a similar fall in both force and stiffness. However, if ADP release is associated with force production, an elevation of MgADP should produce a decrease in force with little to no change in muscle stiffness. To determine if there are differences in the distribution of the cross-bridge states that could contribute to the mechanical differences between phasic and tonic smooth muscle, we characterized phasic and tonic smooth muscles by using increasing multi-sine-wave length perturbations of muscle length during the steady state for relaxed, activated, and rigor states of permeabilized smooth muscle strips. We then examined the changes in force and stiffness that occur when the relative distributions of cross-bridge states are perturbed with the elevation of P_i or MgADP.

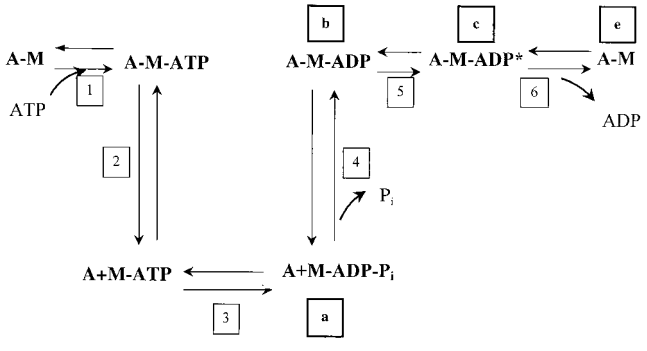

FIGURE 1 Proposed cross-bridge model for smooth muscle (Fuglsang et al., 1993), as adopted from Marston and Taylor (1980) and Hibberd and Trentham (1986).

MATERIALS AND METHODS

Muscle preparation

The portal vein and aorta were removed from 4–6- kg adult New Zealand white rabbits. The smooth muscles were quickly removed, rinsed in physiological saline solution several times, and stored in oxygenated, 4°C physiological saline solution for up to 8 h. Fat and connective tissue were removed, and small smooth muscle strips (~0.75 × 1.0 mm) were dissected from the portal vein and aorta and attached to aluminum T-clips (Goldman and Simmons, 1984). The portal vein and aortic membranes were chemically permeabilized in relaxing solution containing 1% Triton (Sigma UltraPure; Table 1) at 25°C for 45 and 105 min, respectively. The permeabilized tissue was removed from the detergent and rinsed with relaxing solution before it was mounted on the experimental apparatus.

The experimental apparatus

Measurements of fiber mechanics were made on an experimental apparatus similar to that described by Smith and Barsotti (1993). The muscle was mounted between a solid-state force transducer (AE801; Sensonor, Horten, Norway) and a piezoelectric stack length driver (Physiks Instruments, Walbronn, Germany). Two small hooks were fashioned from 50-μm-diameter stainless steel rods and connected to both the force transducer and the piezoelectric length driver. The length driver was controlled through an amplifier interfaced with a computer, using a National Instrument D/A and A/D data acquisition board (PCI-MIO-16XE-10; 100 kS/S, 16-bit; National Instruments). The frequency response of the force transducer, hooks, and attached muscle was 2 kHz. The tissue was bathed in 200-μl wells of a rotating Teflon platform, and the solutions were changed using a stepped, optical feedback-looped motor. The length driver, force transducer, and rotating stage were mounted on an aluminum platform on a vibration isolation table (Micro-G; TMC, Peabody, MA) inside an electrically grounded cage.

Increasing frequency sinusoidal sequence

The length perturbation sequence combined 4 cycles/frequency of a 5.3-μm (0.7% total tissue length, L_o) peak-to-peak sine wave ramping from 1 to 100 Hz in 1-Hz increments. The end of each series was padded with 0.36 s of zeroes to create a total sequence length of 14 s. After Fourier transformation, the sequence yielded a frequency response with a 100-Hz bandwidth at 1-Hz resolution (Shue and Brozovich, 1999). The sine waves

TABLE 1 Composition of solutions for permeabilized portal vein and aorta tissue studies (mM)

	Relaxing	Activating	Rigor	ATPγS-relaxing	ADP-activating	P_i-activating	Rigor apyrase
Ca^{2+}	0.02	5.26	0.02	0.02	5.32	5.25	0.02
Mg^{2+}	1.00	1.00	1.00	1.00	1.00	1.00	1.00
CP	25.00	25.00	0.00	0.00	0.00	0.00	0.00
MgATP	5.00	5.00	0.00	0.00	5.00	5.00	0.00
ADP	0.00	0.00	0.00	0.00	5.00	0.00	0.00
P_i	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MgATPγS	0.00	0.00	0.00	1.00	0.00	0.00	0.00
Apyrase	0.00	0.00	0.00	17.1 U	0.00	0.00	17.1 U
EGTA	5.00	5.00	5.00	5.00	5.00	5.00	5.00
KMS	69.24	58.61	171.22	165.59	113.53	109.10	171.22
BES	25.00	25.00	25.00	25.00	25.00	25.00	25.00

Concentrations of individual components are free concentrations and are calculated as described in Brozovich and Yamakawa (1995), using binding constants per Andrews et al. (1991). Calmodulin (5 μM) was added to all solutions.

were generated at 4 kHz, and the resultant force and length of the tissue during length perturbation were sampled at 4 kHz. Data were collected during the steady state of the force response after solution changes. The length perturbation sequence was continuously applied for 14 s and averaged over five consecutive trials to reduce the influence of noise on the frequency response of muscle stiffness measurements.

For both preparations (portal vein and aorta), L_o averaged 130% of the resting length (data not shown), and thus in these experiments, the preparations were stretched to L_o. We have shown that stiffness is constant for length changes between 0.5% and 2.0% of L_o in tissue strips, and at length changes larger than 2% L_o, force and stiffness fell, suggesting that the perturbations detached cross-bridges (data not shown). Similar experiments in single smooth muscle cells show that stiffness is constant for length changes smaller than 1.3% of cell length (Shue and Brozovich, 1999). In addition, the intercept of stiffness determined from quick release experiments in single smooth muscle cells has been reported to be ~1.5% (Warshaw and Fay, 1983), further suggesting that a 0.7% oscillation would not result in the detachment of attached cross-bridges. For each experiment, force and stiffness in relaxing solution were set at zero and used as the reference for the frequency response of all other states.

Chemicals and solutions

Solutions and calcium buffers were mixed according to a computer program that calculates a given set of free ion concentrations for the amount of stock solutions to be mixed (Brozovich and Yamakawa, 1995). The binding constants for the ionic species were corrected for both temperature and ionic strength (Andrews et al., 1991); the composition of the solutions is listed in Table 1. The solutions were adjusted to pH 7.1, with a final ionic strength of 200 mM. All experiments were performed at room temperature (22°C).

RESULTS

Relaxed state

The relaxed state was obtained after stretching the tissue to L_o, changing the well solution three times with fresh relaxing solution, and allowing the preparation to equilibrate. After steady state was reached, force and stiffness were determined as a function of frequency (see Materials and Methods). The relaxed state was characterized by low force and low stiffness for both the portal vein and aorta (Fig. 2) and is consistent with previously published reports (Martin and Barsotti, 1994; Khromov et al., 1996). It has been suggested that attached cross-bridges are present in relaxed smooth muscle. To investigate this possibility we used ATP-γ-S, a nonhydrolyzable ATP analog that shifts the population of cross-bridges to the detached A+M-ATP-γ-S state (Goody et al., 1980). The ATPase/ADPase apyrase (Barsotti and Ferenczi, 1988; Nishiye et al., 1993) was also added to the ATP-γ-S relaxing solution to remove contaminating endogenous ATP and ADP from permeabilized fibers. Using thin-layer chromatography, we demonstrated that a 5-min treatment of ATP-γ-S relaxing solution with apyrase eliminated contaminating ADP but did not hydrolyze ATP-γ-S (data not shown). Upon transfer from relaxing solution to ATP-γ-S relaxing solution with apyrase, the portal vein did not exhibit a significant change in stiffness ($-1 \pm 1\%$, $n = 4$, Fig. 3 *a*). However, in the aorta, the transfer from relaxing solution to ATP-γ-S relaxing solution with apyrase decreased stiffness ($-33 \pm 4\%$, $n = 3$, Fig. 3 *b*), suggesting that for aortic smooth muscle in relaxing solution, there exists a population of attached cross-bridges.

Rigor state

The rigor (A-M) state was achieved by incubating the portal vein or aorta in rigor solution for at least 1 h while changing to fresh rigor solution every 15 min. After this protocol in rigor solution, stiffness was determined for the portal vein and aorta. As reported by others (Kawai and Brandt, 1980), we expected the rigor (A-M) state to be characterized by a constant stiffness-versus-frequency profile over the measured frequencies. The portal vein demonstrated a flat and constant stiffness profile. On the other hand, the aorta did not display these "rigor" characteristics; but instead, stiffness was not constant for all frequencies of oscillation. This suggests that for the aorta in rigor solution, not all cross-bridges populate the rigor (A-M) state. To test for the presence of a cross-bridge population other than A-M in rigor solution for both the portal vein and aorta, we utilized

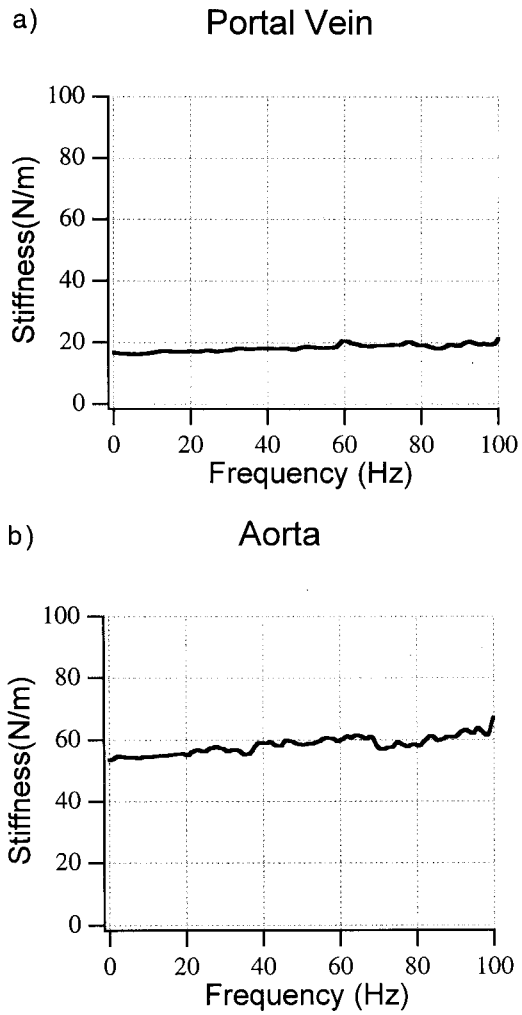

FIGURE 2 Stiffness profiles of rabbit portal vein and aorta from 1 to 100 Hz for 1% Triton-permeabilized tissue strips in relaxing solution (pCa = 9). These stiffness traces represent the relaxed state for the respective tissues and were referenced as 0. All other states are represented as the change from the relaxed state.

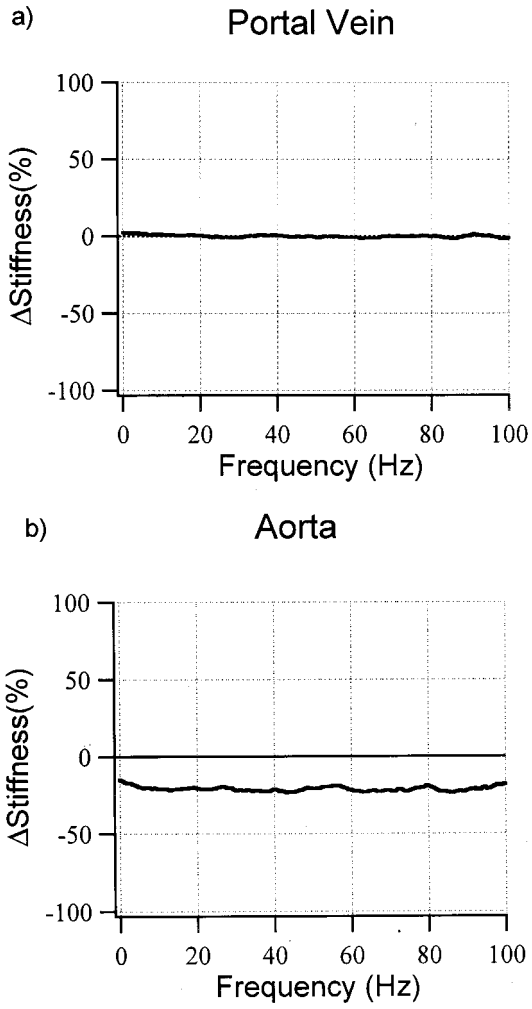

FIGURE 3 Representative traces of the percentage change in stiffness for the portal vein and aorta as the tissue is transferred from relaxing solution to ATP-γ-S relaxing solution. The portal vein stiffness in relaxing solution did not significantly change when incubated in ATP-γ-S relaxing solution, while aortic stiffness decreased 20%.

apyrase. Apyrase catalyzes the hydrolysis of any endogenous ATP and/or ADP in the permeabilized muscle strips when bound to cross-bridges. In the portal vein, there was no significant differences in stiffness ($-3 \pm 1\%$, $n = 4$, Fig. 4 *a*) between rigor solution with apyrase and rigor solution. On the other hand, in the aorta, the addition of apyrase to the rigor solution increased stiffness ($+22 \pm 3\%$, $n = 4$, Fig. 4 *b*) compared to rigor solution. These results support the idea that for the aorta, in rigor solution, the cross-bridge population is mixed, consisting of both A-M-ADP and A-M states.

Active state

Compared to the relaxed baseline, calcium activation of portal vein and aorta significantly increased stiffness from a resting level of 17.5 ± 2.4 N/m ($n = 8$) to 66.3 ± 4.1 N/m ($n = 10$) in the portal vein and from 47.5 ± 4.2 N/m ($n = 7$) to 96.1 ± 6.7 N/m ($n = 9$) in the aorta (Fig. 5). This is consistent with an increase in the number of actively cycling, force-producing cross-bridges and is similar to reports by others for skeletal (Goldman and Simmons, 1984), cardiac (Barsotti and Ferenczi, 1988), and smooth (Somlyo et al., 1988) muscle.

Phosphate release step and its association with force production

The effect of increasing the relative population of the pre-phosphate release state (Fig. 1, *state a*) in portal vein and aorta was analyzed by measuring the change in force and stiffness when the tissue preparation was transferred from

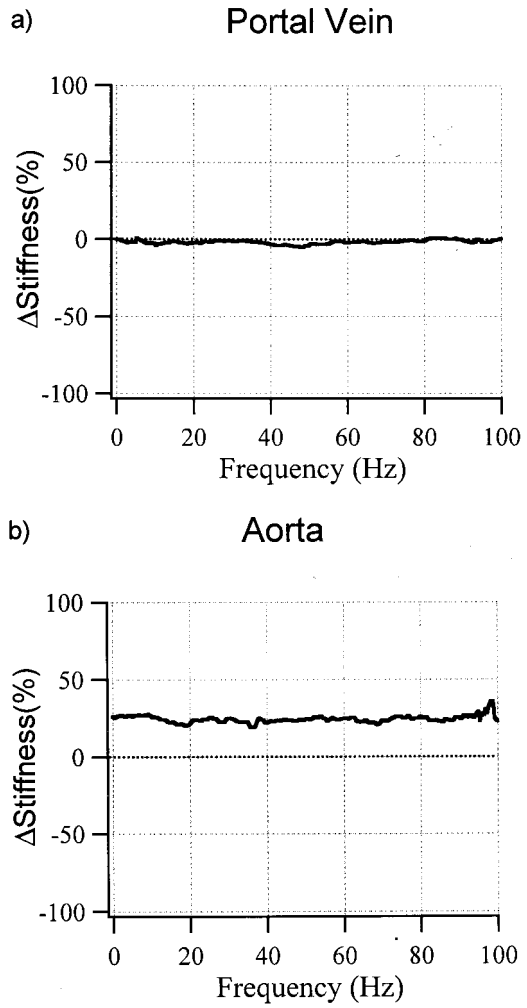

FIGURE 4 Representative traces of the percentage change in stiffness for the portal vein and aorta as the tissue is transferred from rigor solution to rigor solution containing 17.1 units of apyrase. Portal vein stiffness in rigor solution did not change significantly when incubated in the presence of apyrase, while in this example the aorta stiffness increased by 25%.

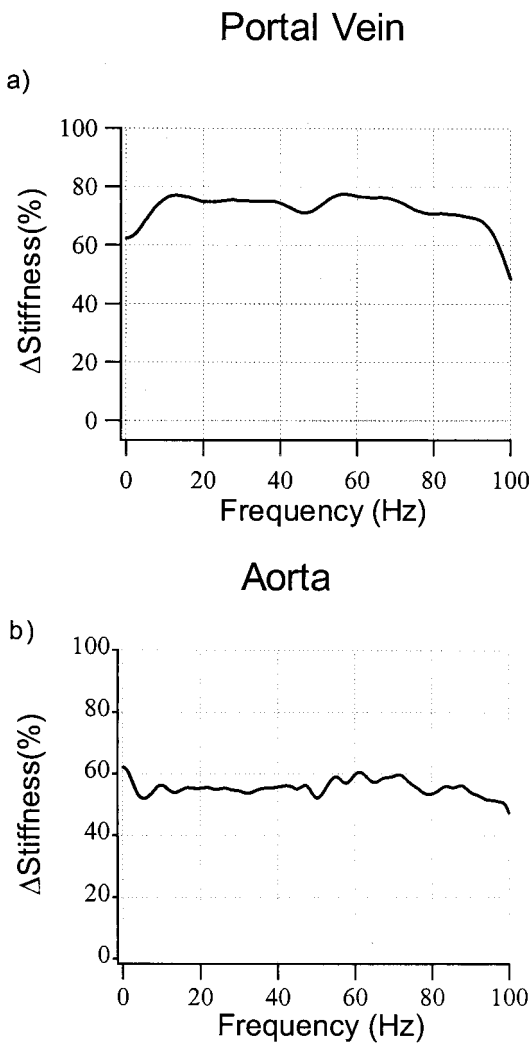

FIGURE 5 Change in stiffness for the portal vein and aorta from the relaxed state (pCa = 9) upon calcium activation (pCa = 4).

activating solution to activating solution containing 12 mM inorganic P_i. The increase in inorganic P_i resulted in a decrease in force for both the portal vein ($-42 \pm 1\%$, $n = 4$, Fig. 6 *a*) and aorta ($-30 \pm 1\%$, $n = 3$, Fig. 7 *a*). In addition for the portal vein, stiffness also decreased ($-40 \pm 4\%$, $n = 4$, Fig. 6 *b*), while in the aorta stiffness similarly dropped ($-18 \pm 1\%$, $n = 4$, Fig. 7 *b*). These results are consistent with several published reports, which demonstrate a decrease in force with the elevation of inorganic P_i (Osterman and Arner, 1995; He et al., 1998).

ADP release step and force production

To investigate the role of ADP release in the cross-bridge cycle and force production for portal vein and aorta, the relative population of pre-ADP release cross-bridge states (Fig. 1, *states b* and *c*) was increased using 5 mM MgADP. In portal vein, when the preparation was moved from activating solution to activating solution with 5 mM MgADP, steady-state force decreased slightly ($-2 \pm 1\%$, $n = 6$, Fig. 8 *a*). Surprisingly, there was a large decrease ($-50 \pm 3\%$, $n = 6$, Fig. 8 *b*) in stiffness, suggesting the detachment of actin-bound, low-force-producing cross-bridges. However, in the aorta, transfer from activating solution to activating solution with 5 mM MgADP decreased force ($-30 \pm 3\%$, $n = 3$, Fig. 9 *a*) and stiffness ($-28 \pm 3\%$, $n = 3$, Fig. 9 *b*), suggesting that the ADP release step is associated with force production.

To determine whether the affect of 5 mM MgADP on force and stiffness in the aorta was due to only the elevation of MgADP and not to other factors, we performed two different control experiments. First, we assessed the possibility that the MgADP effect is reversible. When the aorta

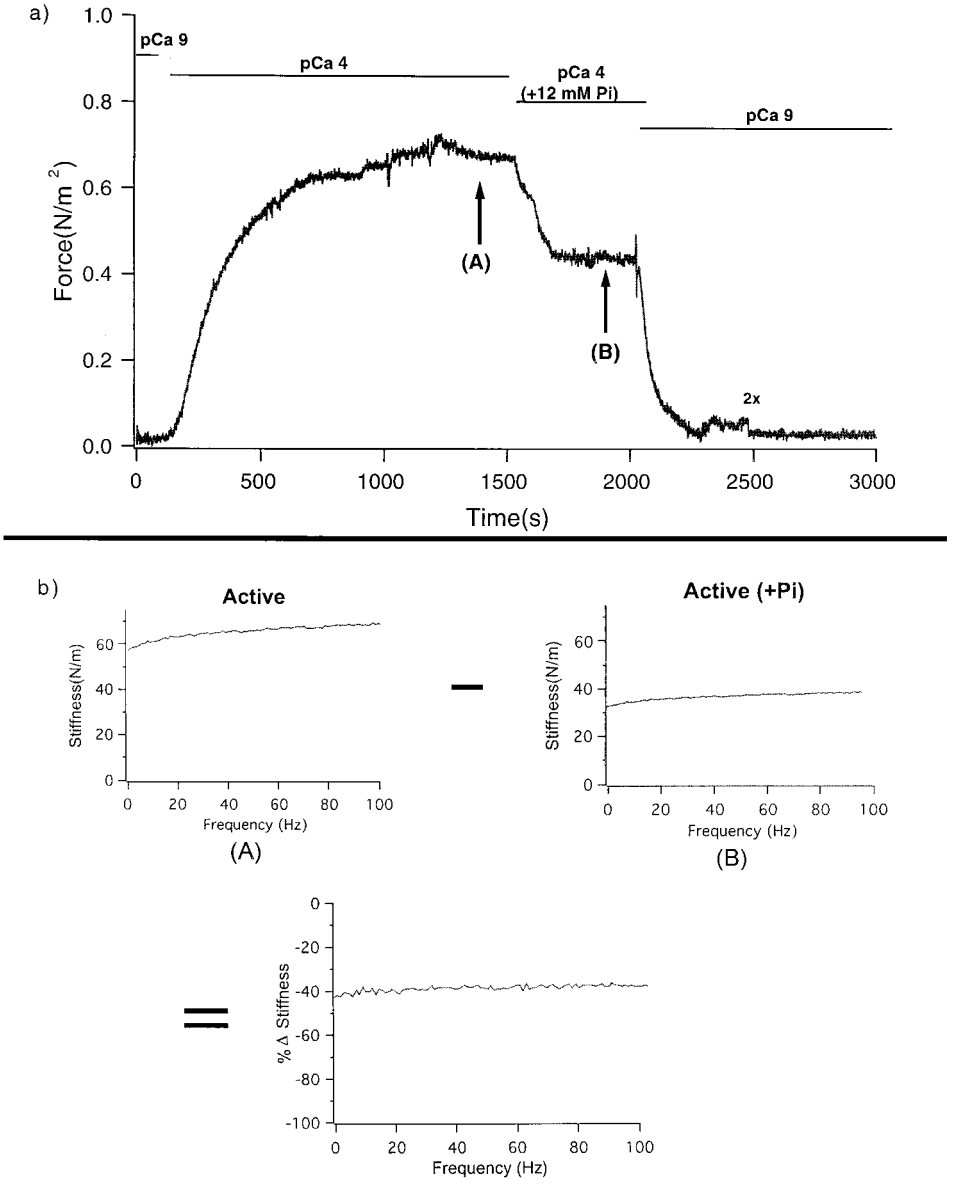

FIGURE 6 Force and stiffness profile upon transfer of portal vein from activating solution to activating solution with 12 mM inorganic P_i. (*a*) Force profile of rabbit portal vein upon transfer from relaxing solution to activating solution, then to activating solution with 12 mM inorganic P_i, and back to relaxing solution. There was a decrease ($-42 \pm 1\%$, $n = 4$) in force when the portal vein strip was transferred from activating solution to activating solution with 12 mM P_i. (*b*) Concurrent with the decrease in force from activating solution to activating solution with 12 mM P_i, stiffness decreased ($-40 \pm 4\%$, $n = 4$), suggesting that 12 mM P_i results in the detachment of attached, force-producing cross-bridges. Stiffness data were taken at the time points marked by arrows (A) and (B) in *a*.

was transferred from activating solution with 5 mM MgADP to activating solution (Fig. 10 *a*), the aorta redeveloped force back to the original maximum Ca^{2+} activated level ($n = 4$), which demonstrates that the MgADP effect is reversible. Second, to show that this effect was not due to a change in MLC_{20} phosphorylation, the skinned aortic strips were first thiophosphorylated with ATP-γ-S. After thiophosphorylation, the preparations were activated with MgATP, and force rapidly increased (Fig. 10 *b*). After force reached a steady state, the tissue strip was transferred to activating solution with 5 mM MgADP and force fell ($-26 \pm 1\%$, $n = 4$), an effect similar to that obtained with nonthiophosphorylated preparations. Again, this MgADP effect was reversible with force redevelopment when the preparation was transferred from activating solution with 5 mM MgADP to activating solution.

DISCUSSION

Relaxed state

When the tissue strips were transferred from the relaxing solution to ATP-γ-S relaxing solution with apyrase, stiffness and force did not change for the portal vein but decreased for the aorta. These data suggest that in the aorta, a population of attached cross-bridges exists in relaxing solution. Because ATP-γ-S is a nonhydrolyzable analog of ATP (Goody et al., 1980), substitution of ATP-γ-S for ATP would detach any attached cross-bridges present in relaxing solution and result in an increase in the population of detached, A+M-ATP-γ-S cross-bridges. Detachment of cross-bridges by ATP-γ-S in relaxing solution would decrease overall stiffness, with force decreasing if these cross-bridges were also in a force-producing state(s).

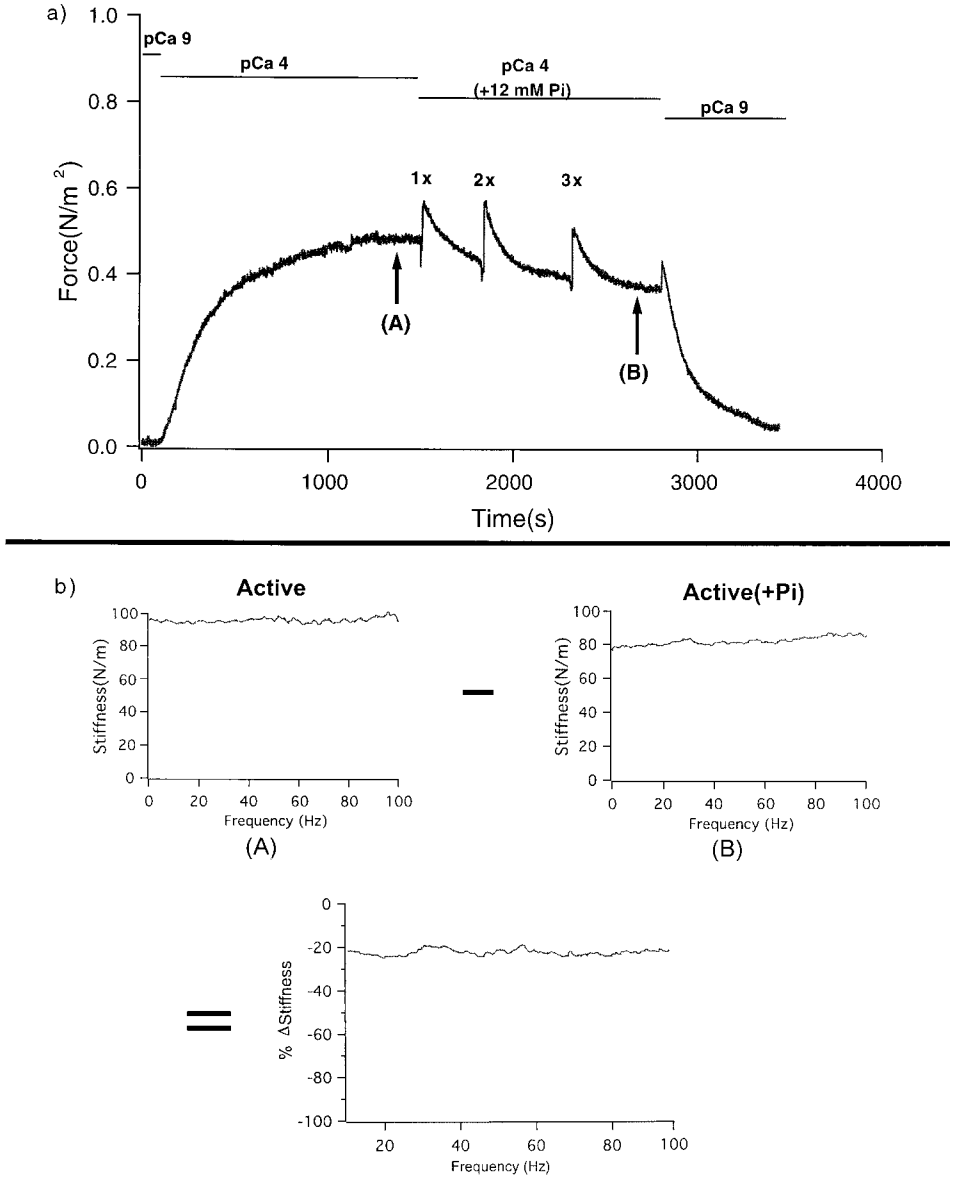

FIGURE 7 Force and stiffness profile upon transfer of aorta from activating solution to activating solution containing 12 mM P_i. (*a*) Force profile of rabbit aorta upon transfer from relaxing solution to activating solution, and from activating solution to activating solution with 12 mM P_i. There was a decrease ($-30 \pm 1\%$, $n = 3$) in force when the aorta strip was transferred from activating solution to activating solution with 12 mM P_i. 1×, 2×, and 3× indicate changes in the solution. (*b*) Concurrent with the decrease in force from activating solution to activating solution containing 12 mM P_i, stiffness decreased ($-18 \pm 1\%$, $n = 4$), suggesting that 12 mM P_i results in the detachment of attached, force-producing cross-bridges. Stiffness data were taken at time points marked by arrows (A) and (B) in *a*.

The existence of a population of attached cross-bridges in the relaxed state has been proposed by previous work from this laboratory (Shue and Brozovich, 1999). This previous investigation demonstrated that in single portal vein cells, the phase angle was consistent with the presence of an energy-generating process in the relaxed state. However, in portal vein tissue strips, we did not detect a change in the stiffness when the preparation was moved from relaxing to ATP-γ-S relaxing solution (Fig. 3 *a*). Nevertheless, in aortic tissue we were still able to detect changes in the stiffness when the skinned aortic strip was transferred from relaxing solution to ATP-γ-S relaxing solution (Fig. 3 *b*). Therefore in the aorta, the data suggest that there is a larger population of attached cross-bridges in comparison to the portal vein in relaxing solution. Because previous work from this laboratory has demonstrated that attached cross-bridges are present in relaxing solution for single portal vein cells, we suggest that for ATP-γ-S relaxing solution, the lack of change in stiffness for the portal vein compared to the aorta could be attributable to a smaller population of attached cross-bridges in relaxing solution.

It has been shown previously that MLC_{20} phosphorylation is required for detached cross-bridges to attach and produce force (Brozovich and Yamakawa, 1995). However, if a population of attached cross-bridges already exists in relaxing solution, these cross-bridges can produce force by moving through a force-generating step and consequently increasing force without increasing MLC_{20} phosphorylation (Brozovich and Yamakawa, 1995; Shue and Brozovich, 1999). The existence of a population of attached cross-

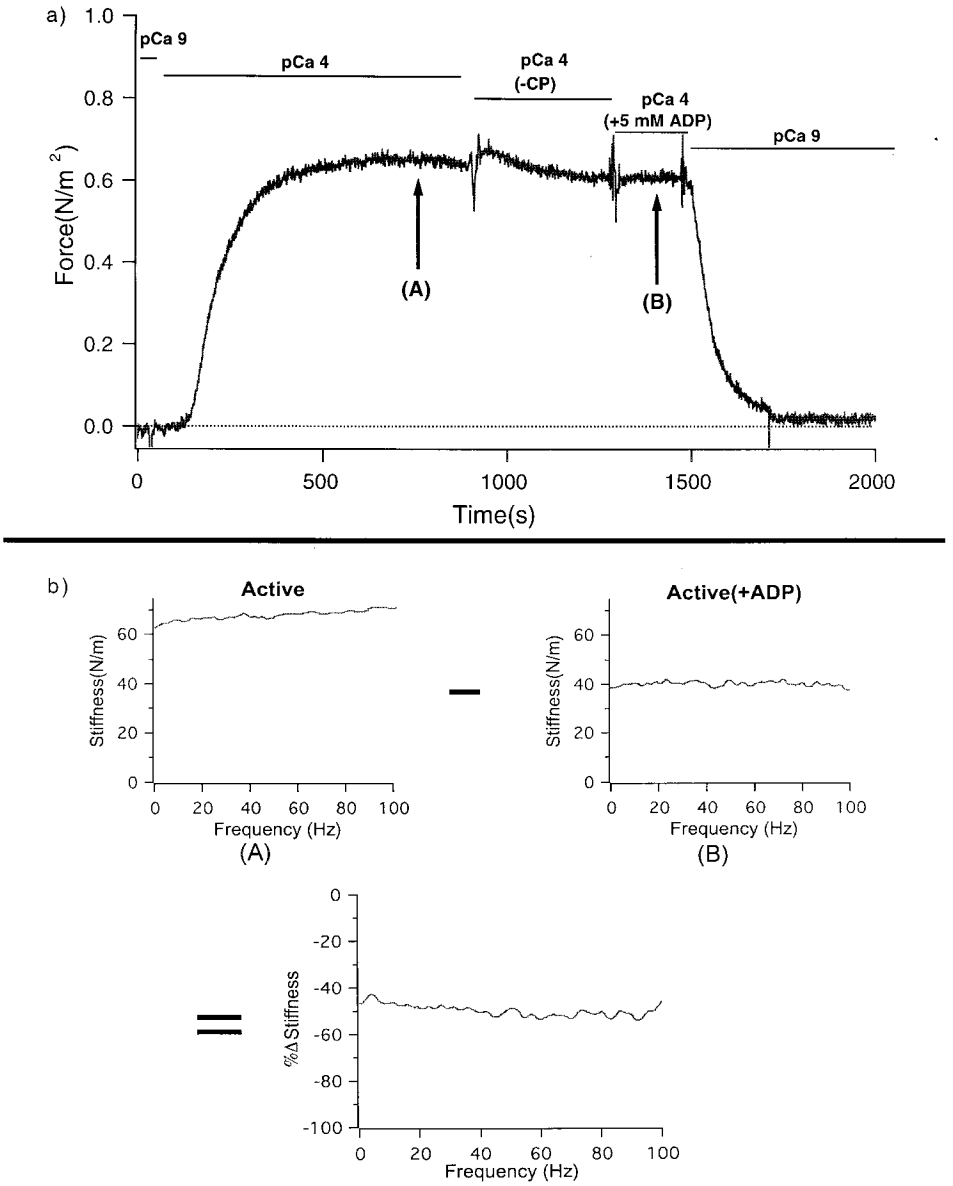

FIGURE 8 Force and stiffness profile upon transfer of portal vein from activating solution to activating solution with 5 mM MgADP. (*a*) Force profile of rabbit portal vein upon transfer from relaxing solution to activating solution, from activating solution to activating solution (-CP), and from activating solution (-CP) to activating solution with 5 mM MgADP. For both portal vein and aorta, changing from activating solution or activating solution (-CP) to activating solution with 5 mM MgADP did not change the results. There was a small decrease in force ($-2 \pm 1\%$, $n = 6$) when the portal vein strip was transferred from activating solution (-CP) to activating solution with 5 mM MgADP. (*b*) A decrease in stiffness ($-50 \pm 3\%$, $n = 6$) was observed when the portal vein strip was transferred from activating solution to activating solution with 5 mM MgADP. Stiffness data were taken at time points marked by arrows (A) and (B) in *a*.

bridges could be the mechanism for those contractions that occur without changes in MLC_{20} phosphorylation, such as stimulation by phorbol esters (Jiang and Morgan, 1987) and sodium hydrosulfite (Yu et al., 1998).

Rigor state

Several other investigators have shown the existence of a population of A-M-ADP cross-bridges (either the A-M-ADP or A-M-ADP* state) in rigor solution for both cardiac (Martin and Barsotti, 1994) and smooth (Nishiye et al., 1993) muscle. When the portal vein is transferred from rigor solution to rigor solution with apyrase, the lack of change in stiffness (Fig. 4 *a*) suggests that the portal vein in rigor solution has a large population of cross-bridges in the rigor (A-M) state. However, in the aorta, the change from rigor solution to rigor solution with apyrase resulted in an increase in stiffness (Fig. 4 *b*). These data suggest that for the aorta, a population of A-M-ADP and/or A-M-ADP* cross-bridges (Fig. 1) exists in rigor solution. Apyrase hydrolyzes ADP and shifts the ADP-bound cross-bridges toward the rigor (A-M) state, resulting in an increase in force as A-M-ADP moves through the force-producing isomerization to A-M-ADP* before entering the A-M cross-bridge state (Fig. 1, *steps 5* and *6*). Furthermore, the overall increase in stiffness suggests that the apyrase treatment increases the total number of attached cross-bridges.

These apyrase results demonstrate that in rigor solution for the aorta, there exists a significant percentage of cross-bridges in the ADP-bound states (A-M-ADP and/or A-M-

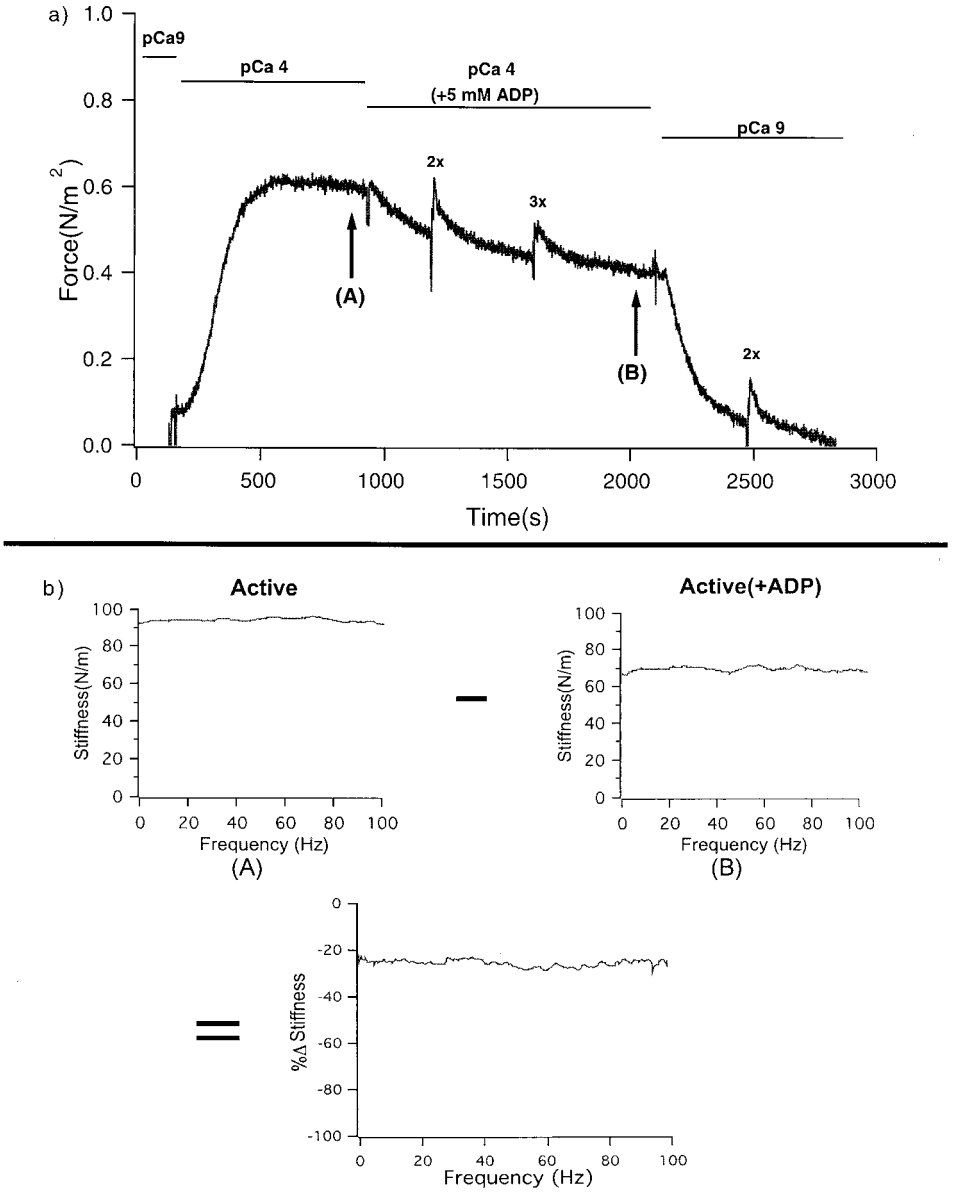

FIGURE 9 Force and stiffness profile upon transfer of rabbit aorta from activating solution to activating solution with 5 mM MgADP. (*a*) Force profile of aorta upon transfer from relaxing solution to activating solution, and from activating solution to activating solution with 5 mM MgADP. There was a decrease ($-30 \pm 3\%$, $n = 3$) in force when the aorta strip was transferred from activating solution to activating solution with 5 mM MgADP. 2× and 3× indicate subsequent changes to fresh activating solution containing 5 mM MgADP. (*b*) Concurrent with the decrease in force with the transfer from activating solution to activating solution with 5 mM MgADP, stiffness decreased ($-28 \pm 3\%$, $n = 3$). Stiffness data were taken at time points marked by arrows (A) and (B) in *a*.

ADP*). The existence of a significant population of ADP-bound cross-bridges in rigor solution for aorta compared to portal vein could be due to the difference in ADP affinity and disassociation between phasic and tonic tissue (Arner et al., 1987; Somlyo et al., 1988; Nishiye et al., 1993). The isoforms of MLC_{17} have been correlated with the relative sensitivities of the different smooth muscles types to MgADP (Fuglsang et al., 1993). The concentration of the more basic MLC_{17} isoform was highest in tonic tissue, which also has the highest affinity for MgADP. In contrast, phasic tissue predominantly expresses the acidic MLC_{17} isoform, which has a lower affinity for MgADP (Fuglsang et al., 1993), faster shortening velocities (Malmqvist and Arner, 1991), faster rates of force development (Horiuti et al., 1989), and higher ATPase activity (Hasegawa and Morita, 1992). In addition, the differences in the 7-amino acid insert near the ATP binding site, which differs in phasic and tonic smooth muscle myosin heavy chains (Kelley et al., 1993; White et al., 1993), may also affect MgADP affinity in phasic and tonic smooth muscle. Therefore, in tonic tissue, a lower ADP K_d would result in a larger population of MgADP-bound cross-bridges (A-M-ADP and/or A-M-ADP*). ADP disassociation is higher in the portal vein compared to the aorta (Khromov et al., 1996), which may produce a nondetectable population of ADP-bound cross-bridges in rigor solution. Others (Sweeney et al., 1998) have also suggested that for tonic smooth muscle, a lower K_d for ADP would result in a slower cycling rate compared to phasic smooth muscle. This could partially explain the slower force generation and

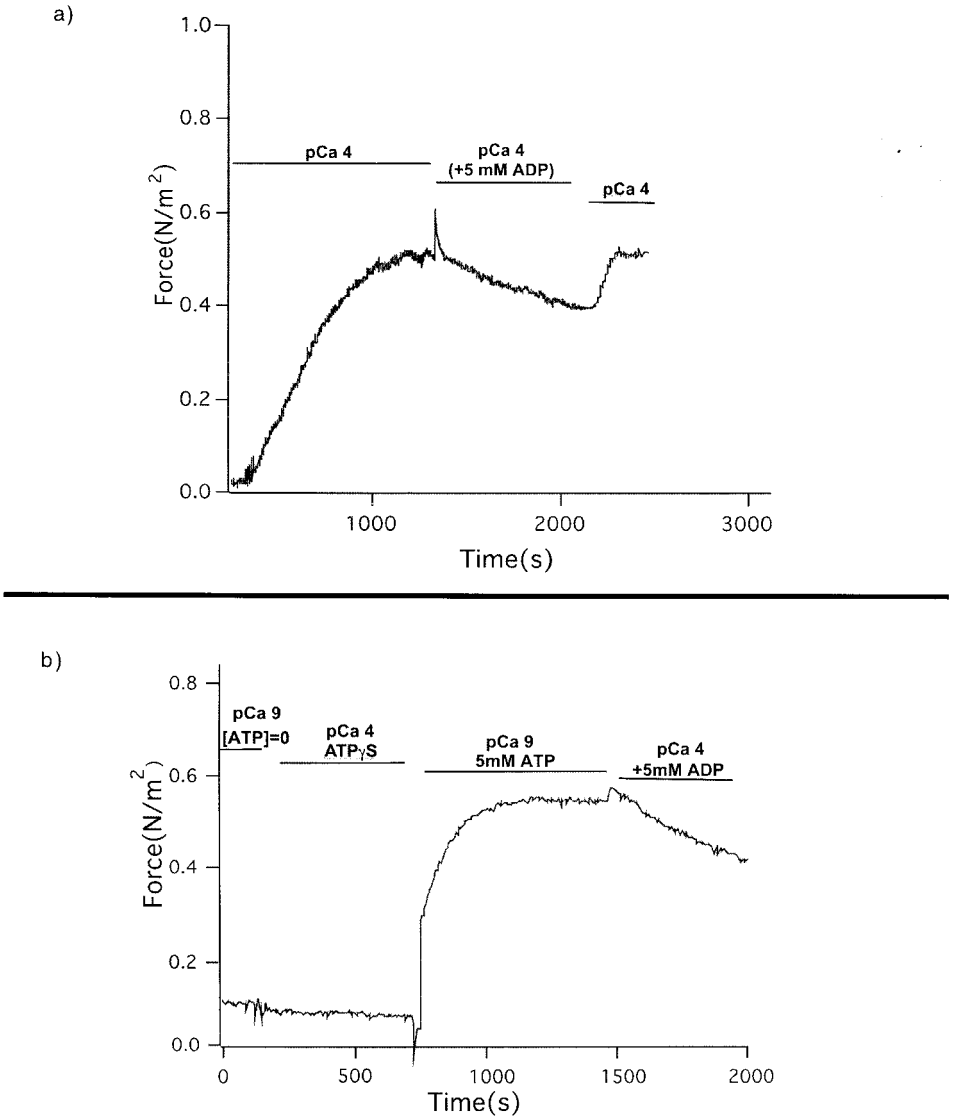

FIGURE 10 (*a*) Force profile upon transfer of rabbit aorta from relaxing to activating solution, from activating solution to activating solution with 5 mM MgADP, and back to activating solution. Force increased to the original maximum force when aorta was transferred from activating solution with 5 mM MgADP to activating solution ($n = 4$). (*b*) Force profile upon transfer from ATP-γ-S rigor solution to activating solution, and from activating solution to activating solution with 5 mM MgADP solution. Force decreased ($-30 \pm 3\%$, $n = 5$) upon exposure to 5 mM MgADP, which is similar to the ADP response for non-thiophosphorylated tissue.

lower ATPase activity of tonic compared to phasic smooth muscle.

Active state

The large increase in force and stiffness (Fig. 5) for both the portal vein and aorta is consistent with an increase in the number of cycling, force-producing cross-bridges during activation. As expected, force production was faster in the portal vein than in the aorta, consistent with their phasic and tonic properties, respectively. Similar increases in force and stiffness have been reported by others (Somlyo et al., 1988).

Phosphate release

Phosphate release has been shown to be regulated by MLC_{20} phosphorylation (Sellers, 1985) and, in smooth muscle, is thought to occur concurrently with or just before cross-bridge attachment and force generation (Fig. 1, *step 4*). Thus inhibition of P_i release should inhibit cross-bridge attachment and increase the relative population of the A+M-ADP-P_i state (Fig. 1, *state a*). If inhibition of P_i release occurs with the elevation of P_i, one would predict that force and stiffness should fall proportionally. Similar to studies in skeletal (Kawai et al., 1987; Pate et al., 1998), cardiac (Barsotti and Ferenczi, 1988), and smooth (Itoh et al., 1986; Osterman and Arner, 1995) muscle, we have demonstrated that elevating P_i decreases force and stiffness. Our results for both the portal vein (Fig. 6) and aorta (Fig. 7) suggest that elevation of P_i decreases steady-state force and stiffness by increasing the relative population of the weakly bound or detached A+M-ADP-P_i state.

Others (Brozovich et al., 1988) have suggested that in skeletal muscle there exists an attached but low-force A-M-

ADP-P_i state in addition to the detached, A+M-ADP-P_i state. If this state exists in smooth muscle, an increase in P_i should shift the population of cross-bridge states and lead to a relative increase in the attached, low-force-producing A-M-ADP-P_i state. An increase in the relative population of an attached but low-force-producing A-M-ADP-Pi state should produce a smaller fall in stiffness than in force. In the portal vein, elevation of P_i produced a proportionate fall in stiffness and force. These results suggest that a low-force A-M-ADP-P_i does not exist for the portal vein (Fig. 1). However, in the aorta, stiffness decreased by 18% while force fell by 30%, a fall in stiffness that is 40% less than the fall in force. This suggests that an attached, low-force-producing A-M-ADP-P_i cross-bridge state may exist in the aorta. Consequently, for tonic smooth muscle, the cross-bridge model in Fig. 1 requires modifications to include this potential state (Fig. 11, *state a.2*).

ADP release

Cooke and Pate (1985) have demonstrated in skeletal muscle fibers that force increases when the fiber is transferred from activating solution to activating solution with elevated MgADP, which suggests that force is produced before ADP release from the cross-bridge. However, recent literature (Whittaker et al., 1995; Gollub et al., 1996; Jontes and Milligan, 1997) suggests that in smooth muscle the binding of MgADP to the cross-bridge is associated with a conformational change in the light-chain domain, resulting in a significant movement of the head-rod junction. This change represents an extra movement of smooth muscle S_1 beyond that of skeletal muscle S_1 (Whittaker et al., 1995; Gollub et al., 1996). If there is force production associated with the extra movement of the S_1 head by ADP release, then elevating MgADP to increase the relative population of the A-M-ADP states should decrease force.

Portal vein

When portal vein tissue was transferred from activating solution to activating solution containing 5 mM MgADP, there was a small decrease in force but a large, ~50% decrease in stiffness (Fig. 8 *b*). Therefore, for the portal vein, our data suggest that ADP release is not a force-producing step (Fig. 1, *step 6*). This correlates with similar reports for other phasic smooth muscle (Khromov et al., 1996) and fits well with the original cross-bridge model scheme presented by Fuglsang et al. (1993). This cross-bridge scheme (Fig. 1) suggests that the force-generating step is an isomerization from the low-force to the high-force A-M-ADP state (Fig. 1, *step 5*). Similar to our results, Dantzig et al. (1999) reported a decrease in stiffness when rigor tissue was exposed to elevated MgADP. The large decrease (~50%) in stiffness we observed when transferring the portal vein tissue from activating solution to activating solution containing 5 mM MgADP suggests that elevated MgADP preferentially affects low-force-producing cross-bridges (Fig. 1, *state b*), leading to their detachment (Fig. 1, *state a*). Detachment of low-force-producing cross-bridges (Fig. 1, *state b*) can be explained by the cross-bridges exiting backward out of the cross-bridge cycle with subsequent detachment (Fig. 1, *reversal of step 4*), or, alternatively, by the existence of a separate, detached A+M-ADP state (not shown), as suggested by the model of Piazzesi et al. (1993). According to Piazzesi et al. (1993), an intermediate, detached state (A+M-ADP) exists that would allow the attached, ADP-bound cross-bridges (Fig. 1, *states b* and *c*) to detach without moving backward to the A+M-ADP-P_i state (Fig. 1, *state a*) and reattach to actin without ADP dissociation. This type of mechanism can be used to explain the multiple steps of the myosin head per hydrolysis of a single ATP (Lombardi et al., 1992; Kitamura et al., 1999).

Aorta

In the aortic preparations, transferring the tissue from activating solution to activating solution with 5 mM MgADP decreased force and stiffness proportionally (Fig. 9), suggesting that the elevation of MgADP decreases force and detaches cross-bridges. These results were surprising, particularly because the release of ADP has not generally been thought to be coupled to a major portion of the powerstroke. However, if the extra 28° angle shift in the smooth muscle

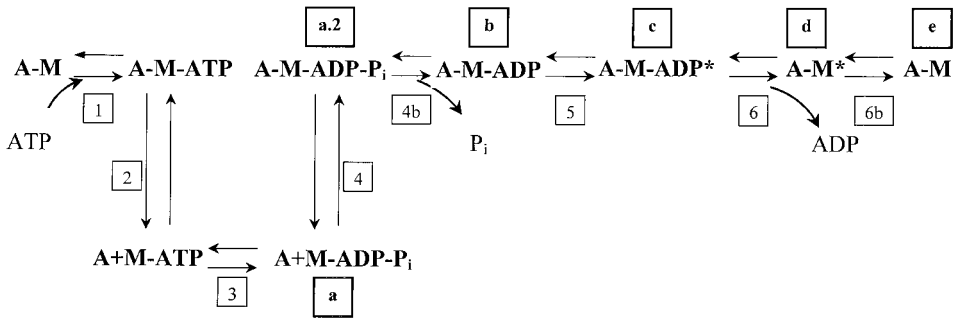

FIGURE 11 Proposed cross-bridge model for tonic smooth muscle as adopted from Fuglsang et al. (1993).

S_1 resulting from MgADP release is associated with a significant portion of the force-producing step(s) (Whittaker et al., 1995; Gollub et al., 1996; Jontes and Milligan, 1997), elevation of ADP concentrations should prevent ADP release and thus result in a fall in force. The explanation for the decrease in stiffness with the transfer from activating solution to activating solution with 5 mM MgADP could be similar to that above for the portal vein: by the cross-bridge exiting backward out the cycle (*reversal of step 4*, Fig. 1) or through a detached A+M-ADP state (Piazzesi et al., 1993).

In the aorta, given that our data suggest that a significant portion of the force-producing step is associated with ADP release, the subsequent rigor (A-M) state has to be a force-producing state (Fig. 1, *state e*). However, ATP-induced detachment from the rigor (A-M) state has been shown to occur at a rate of $\sim 10^5$ M^{-1} s^{-1} (Nishiye et al., 1993; Khromov et al., 1996). At 5 mM ATP, the rate of cross-bridge detachment would occur at 500 s^{-1}. Thus the rigor (A-M) state would be very short lived and produce little or no force. To maintain force, these data suggest that a separate, force-producing rigor state (A-M*; Fig. 11, *state d*) would have to exist that is similar to that suggested by measurements of force and ATPase activity of single myosin S_1 heads (Ishijima et al., 1998).

CONCLUSIONS

The data suggest that for tonic smooth muscle, the ADP affinity for the cross-bridge is higher than that for phasic smooth muscle. Our data also suggest that the force-generating mechanism, specifically the state involved, is not the same for the portal vein and aorta. For portal vein, our data are consistent with force production occurring concurrently with and/or after P_i release, but before ADP release, which fits well with the cross-bridge model presented in Fig. 1 (Fuglsang et al., 1993). For the aorta, our data suggest that an attached, low-force A-M-ADP- P_i state exists and that a significant portion of the cross-bridge power stroke is linked with the ADP release. Thus in the aorta, the cross-bridge model in Fig. 1 must include the attached, low-force A-M-ADP-P_i and the attached, high-force A-M* states (Fig. 11, *states a.2* and *d*, respectively). These differences in cross-bridge properties could account for the mechanical differences between phasic and tonic smooth muscle.

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