Ca^{2+} increases the apparent viscous component of the passive response of cardiac muscle to stretch

Short Title: Ca^{2+} increases myocardial viscosity

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Keypoints:

- Recent studies found that electrical stimulation of skeletal muscle increased muscle stiffness independently of active contraction. This study aimed to determine if calcium activation of cardiac muscle also increased muscle passive stiffness independently of active contraction.
- Mouse demembranated cardiac trabeculae were probed at different calcium levels by inhibiting active contraction using the myosin ATPase inhibitor para-nitroblebbistatin (PNB).
- Muscle force response to stretch followed a viscoelastic pattern, where the peak force increased with faster stretch velocity, but steady-state force remained independent of stretch velocity.
- With active contraction inhibited by PNB, Ca2+ increased the viscous force response to stretch by approximately 3-fold compared to relaxed (low Ca2+) conditions.
- Calcium activation of a passive viscous mechanical phenomenon may play an important role in cardiac muscle properties during both systole and diastole.

ABSTRACT

Recent studies reported that electrical stimulation of skeletal muscle resulted in an increase of muscle stiffness that was independent of active contraction. The goal of this study was to determine if Ca^{2+}-activation of cardiac muscle increased muscle stiffness, independent of active contraction. The passive mechanical response of mouse demembranated cardiac trabeculae to stretch was probed at different calcium levels by inhibiting active contraction using the myosin ATPase inhibitor *para*-nitroblebbistatin (PNB). Myocardial stiffness was assessed by muscle stretches (≈ 20% initial length) using stretch velocities varying over three orders of magnitude. In response to stretch, muscle force rose to a peak and then relaxed toward a lower steady-state level, consistent with the viscoelastic nature of cardiac muscle. Peak force was higher with faster stretch velocity, but the steady-state force was independent of stretch velocity, consistent with the presence of both apparent viscous and elastic components of the stretch response. The major finding of this study was that with active contraction inhibited by PNB, Ca^{2+} increased the viscous force response to stretch by ≈ 3-fold compared to the response measured under relaxed (low Ca^{2+}) conditions. Moreover, there was a sigmoidal relationship between increased viscous force versus Ca^{2+} level, consistent with a regulated response. For cardiac muscle, Ca^{2+}-activation of a viscous mechanical phenomenon may be an important determinant of both systolic and diastolic properties.

Keywords: Titin, Ca^{2+}, stiffness, HFpEF, systole, diastole

New and Noteworthy: Ca^{2+} is well-known to trigger activation of muscle contraction. This study demonstrates a new mechanical role for Ca^{2+} in cardiac muscle involving a 3-fold increase in the apparent viscosity of muscle tissue. Activation of viscosity by Ca^{2+} might significantly affect both contraction and relaxation of cardiac muscle.

INTRODUCTION

Electrical stimulation of frog skeletal muscle was recently reported to increase the resistance of muscle sarcomeres to stretch, in a manner that was independent of active force development by actin-myosin interactions (1). This phenomenon was attributed to effects on the mechanical properties of titin and on titin's interactions with other sarcomeric proteins. Given the importance of cardiac muscle mechanical properties in health and disease (2, 3), the goal of this study was to determine if and how passive mechanical properties of cardiac muscle are sensitive to activator Ca^{2+}. Using demembranated cardiac trabeculae from male and female mice, we measured muscle force during and following muscle stretches that were imposed with a range of velocities. To measure the effect of Ca^{2+} in the absence of force production, we inhibited cross-bridge cycling using *para*-nitroblebbistatin (PNB) (4). As previously reported, we found that the dynamic force response of cardiac muscle to stretch consists of a velocity-sensitive viscous component and a velocity insensitive elastic component (5-8). Importantly we found that the viscous component of the force response was ≈ 3-fold higher at high Ca^{2+} levels compared to relaxed low-Ca^{2+} conditions. In addition, Ca^{2+} activation of viscous stiffness was greater for cardiac muscle from males vs. females. Our results demonstrate that in addition to triggering activation of contraction, Ca^{2+} also increases the apparent viscous resistance to stretch in cardiac muscle. This finding has significant implications for normal cardiac muscle function and potentially, dysregulation during the development of disease.

METHODS

The study was approved by the Animal Care and Use Subcommittee of the San Francisco Veterans Affairs Medical Center and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (Revised 2011). This institution is accredited by the American Association for the Accreditation of Laboratory Animal Care (Institutional PHS Assurance Number is A3476-01).

Demembranated right ventricular (RV) trabeculae: Trabeculae were prepared as we recently described (9) using 12-week old male and female C57BL/6J mice (Jackson Labs). Briefly, hearts were removed from deeply anesthetized mice, placed in cold arrest solution and flushed with a modified Krebs solution (9). A piece of the RV near the tricuspid valve that contained a trabecula was dissected and immersed in ice cold relaxing solution (see below) plus 2% Triton X-100 (Sigma-Aldrich) for 1 hour, washed in ice-cold relaxing solution for 1 hour, then stored at -20°C for up to 2 months in a 1:1 mixture of relaxing solution and glycerol (10, 11).

Solutions:

Ca^{2+}-free relaxing solution (pCa 11) contained (in mM): EGTA 20; MgATP 8; creatine phosphate 12; N,N-bis[2-hydroxyethyl]2-aminoethane sulfonic acid (BES) 100; pH adjusted to 7.1 with KOH, ionic strength adjusted to 200 mM with KCl, and temperature 21°C (12). Preactivating solution was identical but with calcium-buffering reduced by replacing 19.5 mM of EGTA with HDTA (hexamethylenediamine-N,N,NV,NV-tetraacetate) (Fluka). Activating solution (pCa 4.51) contained 20 mM Ca^{2+}EGTA. Relaxing and activating solutions were mixed to obtain solutions with intermediate pCa (13). All solutions contained 1% (v/v) Protease Inhibitor Cocktail P-8340 and 10 IU/mL creatine kinase (Sigma, St. Louis, MO). To inhibit myosin cross-bridges *para*-nitroblebbistatin (PNB) dissolved in DMSO was used (0.3% DMSO final).

Mechanical studies:

A demembranated trabecula (one per mouse) was attached using aluminum t-clips to a force transducer (Model 400, Aurora Scientific, Inc., Ontario, Canada) and a computer-controlled servo-motor in a small glass-bottomed chamber of a Permeabilized Fiber Test System (Model 1400A, Aurora Scientific, Inc., Ontario, Canada) on an inverted microscope with a video system (Model 900B, Aurora Scientific, Inc., Ontario, Canada) to measure sarcomere length using a 40X objective. Temperature was set to 21°C for mechanical studies.

In relaxing solution, the initial muscle length (Lo) was adjusted to set the sarcomere length to 2.0 μm. Trabecula dimensions were measured (Table 1) and used to normalize muscle force to the muscle cross-sectional area (assuming an elliptical cross-section). Trabeculae were subjected to constant velocity stretches from 0.95 Lo to 1.175 Lo with stretch durations of 100s, 10s, 1s, and 0.1s. After each stretch, muscle length was held constant for 30-60s and then briefly reduced to 0.8 Lo to slacken the muscle and establish the zero force level. Between stretches, muscles were equilibrated for 60s at a length of 0.95 Lo.

Maximal Ca^{2+}-activated force (Fmax) was measured by moving the trabecula to pre-activating solution for 60s, activating solution for 6s, and then returned to relaxing solution.

Cross-bridge cycling was inhibited by addition of PNB (50-100 μM) to all solutions. Stretch-hold protocols were repeated in the presence of PNB in relaxing, activating, and intermediate pCa solutions.

The relationship between muscle viscous force ($F\eta$) versus $[Ca^{2+}]$ was fit to the Hill equation: $F\eta = F\eta_{max} \times [Ca^{2+}]^{nH} / ([Ca^{2+}]^{nH} + EC_{50}{}^{nH})$, where $F\eta_{max}$ is the maximum Ca^{2+}-activated viscous force, EC_{50} is the $[Ca^{2+}]$ at which $F\eta$ is 50% of $F\eta_{max}$, and *n*H is the Hill coefficient reflecting the slope of the relationship at EC_{50}.

The relationship between $F\eta$ and stretch velocity (V) was fit to the dose-response relation: $F\eta = F\eta_{min} + V \times (F\eta_{max} - F\eta_{min}) / (V_{50} + V)$, where $F\eta_{min}$ is minimum value of $F\eta$ (in relaxing solution), and V_{50} is the velocity resulting in a half maximal increase of $F\eta$.

Sex differences in muscle stiffness properties: We compared stiffness properties of RV trabeculae from 12-week old adult male vs. female mice. For males, body weight was greater (25.4 ± 1.3 g, n=3), than for females (weight 20.0 ± 0.2 g, n=3, P = 0.015). However, the dimensions of RV trabeculae, and the passive and active forces of unstretched muscles (sarcomere length 2.0 μm) were not different between males and females (Table 1).

Statistical analysis: Data are presented as mean ± SE. Statistical tests were performed using Prism 10 software (GraphPad Software, Inc., La Jolla, CA) with a significance level set at P<0.05.

Table 1. Dimensions and forces for trabeculae used in stiffness measures

	Male	Female	*P*
Dimensions (μm)			
Length	850 ± 139	810 ± 134	NS
Width	97 ± 8.4	131 ± 17.7	NS
Thickness	83 ± 2.5	84.4 ± 3.4	NS
Forces (kPa)			
Passive	0.93 ± 0.01	0.95 ± 0.11	NS
Developed	57.4 ± 1.9	59.8 ± 10.2	NS
PNB-inhibited	7.3 ± 4.4	5.2 ± 3.1	NS

Pooled measurements for trabeculae from males and females (n = 3/group) and t-test comparisons. Muscle length was adjusted to set the sarcomere length to 2.0 μm.

RESULTS

Effect of strain rate on viscous force response to stretch

Figure 1 shows typical records of muscle length, sarcomere length and muscle force for a relaxed trabecula subjected to a linear ramp stretch of 10 s duration. Muscle length was linearly increased from 0.95 Lo to 1.175 Lo, associated with an increase of sarcomere length from ≈ 1.9 μm to 2.3 μm. In line with previous reports, stretch caused force to increase to a peak and then relax to a steady-state level 30-60s after the stretch (5-8). This stress-relaxation did not involve an appreciable change in sarcomere length.

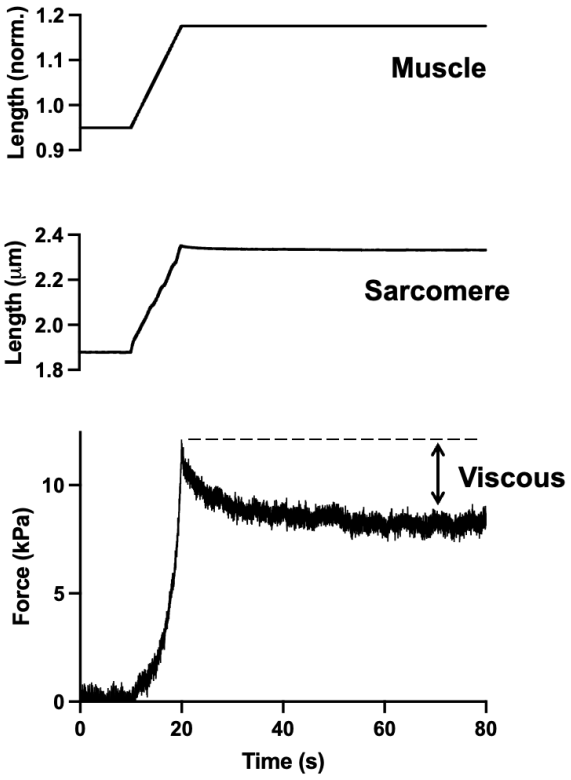


Figure 1. *Example records of changes in muscle length, sarcomere length and muscle force in response to a linear muscle stretch under relaxing conditions. Viscous force of relaxed muscle was quantitated from the difference in the peak force at the end of the stretch versus the quasi-steady state level of force reached 30-60s after the stretch. Muscle length is normalized to the length at which the sarcomere length is 2.0 μm.*

Peak force and steady-state force levels were measured after muscle stretches with velocities varying over three orders of magnitude (Fig. 2A). Peak force and steady-state force were almost identical at the slowest stretch velocity (2.25×10^{-3} muscle lengths per second (ML/s), 100 s stretch duration). However, as previously reported (5), the peak force after stretch progressively increased with increasing stretch velocity (Fig. 2A). In contrast, the steady-state force level (assessed 30s after stretch) did not change as a function of stretch velocity. Accordingly, the viscous force in response to stretch (peak force, minus steady-state force) was observed to increase with increasing stretch velocity in all experiments (Fig. 2B) ($P < 0.0001$, repeated measures one-way ANOVA). The dependence of the viscous force response on stretch speed was not linear and thus is not captured by a linear viscous element model. For all experiments on relaxed muscle, there was not a significant male vs. female difference observed in the effect of stretch velocity on viscous force ($P = 0.72$, repeated measures two-way ANOVA).

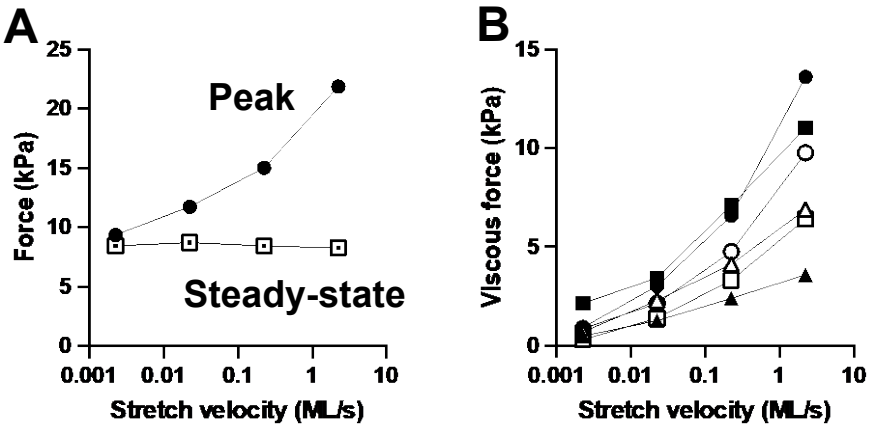


Figure 2. Rapid stretch increased viscous stiffness *(A) Example data of peak and steady state forces levels after stretches of relaxed muscle with velocities ranging over 3 orders of magnitude; note logarithmic abscissa scale for stretch velocity in muscle lengths per second (ML/s). (B) For all muscles in relaxing conditions, the viscous force (peak minus steady state) increased with increasing stretch velocity (P < 0.0001, repeated measures one-way ANOVA).*

The effect of stretch on viscous force in relaxed solution was not different for males (closed symbols) versus females (open symbols) (P = 0.72, repeated measures two-way ANOVA).

Inhibition of myosin cross-bridges by *para*-nitroblebbistatin (PNB)

To determine the effect of Ca^{2+} on the viscous force response, we inhibited Ca^{2+}-activation of actin-myosin cross-bridge formation using PNB (4). In separate dose-finding studies, maximum Ca^{2+}-activated force was substantially decreased by PNB ($EC_{50} \approx 2$ μM, n = 3, Fig. 3A), consistent with previous studies (14). For subsequent experiments, the PNB dose was 50 μM (n=5) or 100 μM (n = 1), consistent with recent studies (15).

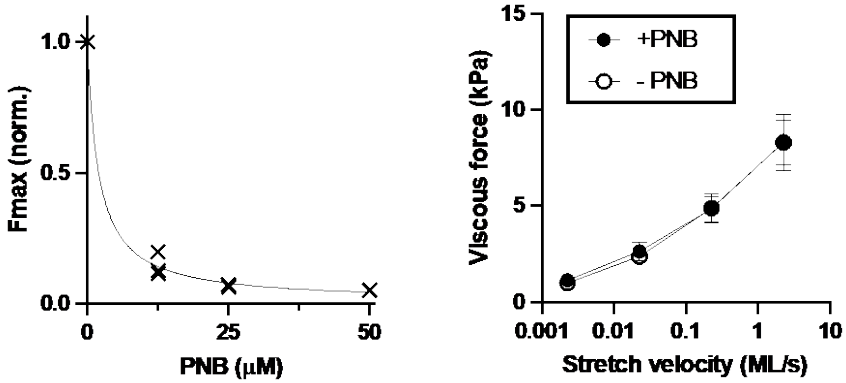


Figure 3. No effect of PNB on viscous stiffness *(A) Dose-response for inhibition by PNB of Ca^{2+}-activated muscle force (normalized to maximum) (n=3). (B) Pooled data of viscous force vs. stretch velocity. Data before PNB treatment (open symbols) were not different compared to after PNB treatment (closed symbols) (P = 0.99, repeated measures two-way ANOVA).*

Inhibition of cross-bridge formation by PNB did not change the viscous force observed in relaxed muscle as a function of stretch velocity (Fig. 3B, *P* = 0.99, repeated measures two-way ANOVA). This finding suggests that in the absence of PNB, cross-bridges do not contribute to the observed viscous component of the force response (e.g. via residual cross-bridge

attachment). Moreover, the reproducibility of the muscle response to stretches before versus after PNB exposure indicates that the processes determining muscle viscosity (e.g. uncoiling and then refolding of titin) are reversible.

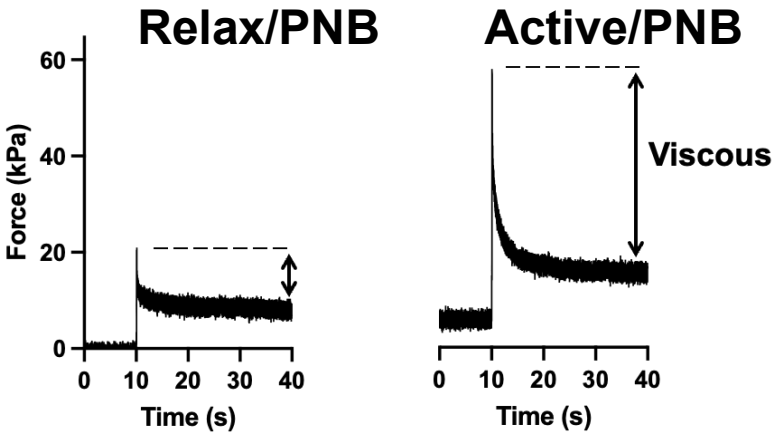


Figure 4. *Example records of changes in muscle force in response to a rapid (0.1 s) stretch in relaxing/PNB solution (left) and activating/PNB solution (right). The viscous component of the force response (arrows) was markedly increased in activating/PNB solution.*

Effect of Ca^{2+} on viscous force response to stretch

Figure 4A shows typical records of changes in force following rapid (0.1s) stretches of a trabecula, first in relaxing solution containing PNB, and then in activating solution containing PNB. The viscous force response to stretch (evidenced by stress-relaxation from peak force to steady-state force) was markedly increased in the activating/PNB solution compared to the relaxing/PNB solution. Figure 4 also shows that the initial level of force prior to stretch was increased in activating/PNB solution, indicating residual activation of contraction. Moreover, the steady-state level of force 30s after the stretch was elevated in activating/PNB solution compared to relaxing/PNB solution, which might also reflect residual activation of contraction.

In the activating/PNB solution, the velocity-dependent, or viscous, component of the force response to stretch was markedly increased at higher stretch velocity in all experiments ($P < 0.0001$, repeated measures one-way ANOVA) (Fig. 5A). Moreover, the viscous force response to stretch was considerably greater in activating/PNB solution compared to relaxing/PNB (Fig. 5B) ($P < 0.0001$, repeated measures two-way ANOVA). The extrapolated maximum in activating/PNB solution (31.7 kPa) was ≈ 3-fold greater than in relaxing/PNB solution (9.1 kPa) (Fig. 5B). However, the stretch velocity that resulted in a half-maximal increase in viscous force was similar in activating/PNB solution (0.23 ML/s) versus relaxing/PNB solution (0.26 ML/s).

Interestingly, the viscous force response to stretch was higher for males than for females (Fig. 5C. $P = 0.039$, 2-way repeated measures ANOVA, n=3 per group). For each muscle, when the viscous force response to stretch was normalized to the maximum developed force, the observed sex difference was maintained and had greater statistical significance ($P = 0.0035$, 2-way repeated measures ANOVA, n=3 per group).

The dependence of the viscous force component on Ca^{2+} level was assessed in terms of the response to the fastest stretch velocity (2.25 ML/s) over a range of Ca^{2+} levels. The viscous force vs. pCa relation (Fig. 6) had a sigmoidal form that was well described by the Hill equation (EC_{50} = 1.4 μM, nH = 2.1, n = 6). This relationship did not differ between males and females ($P = 0.89$, 2-way repeated measures ANOVA).

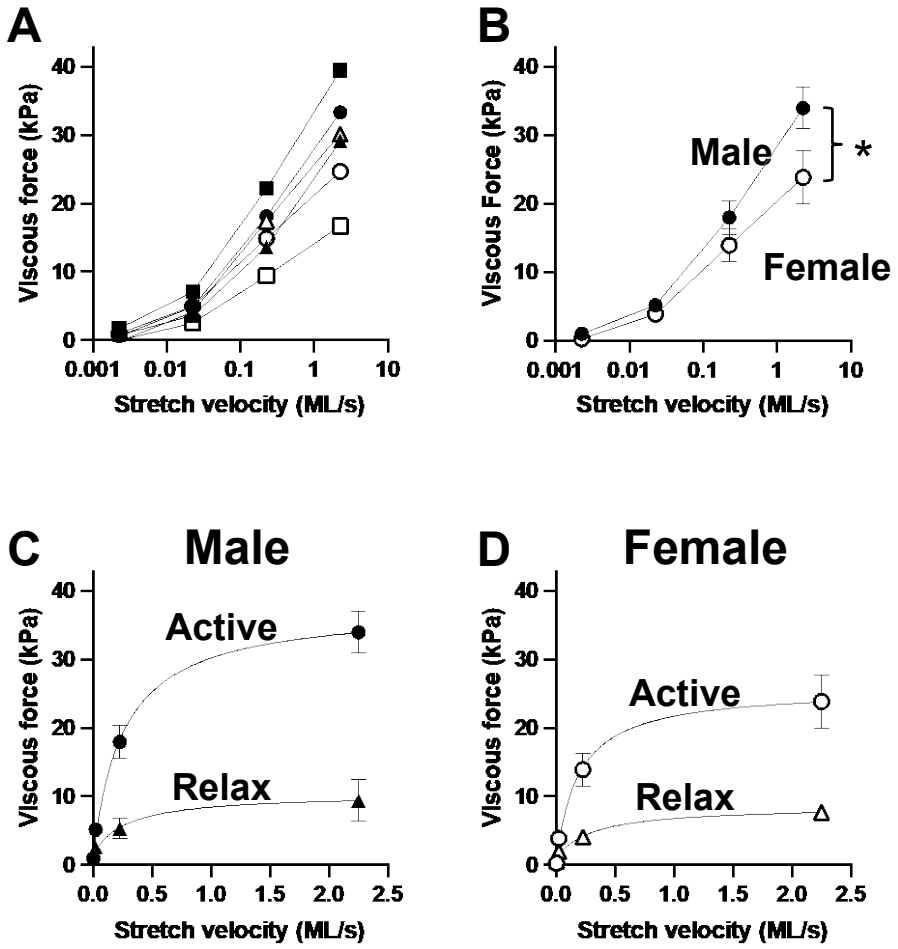
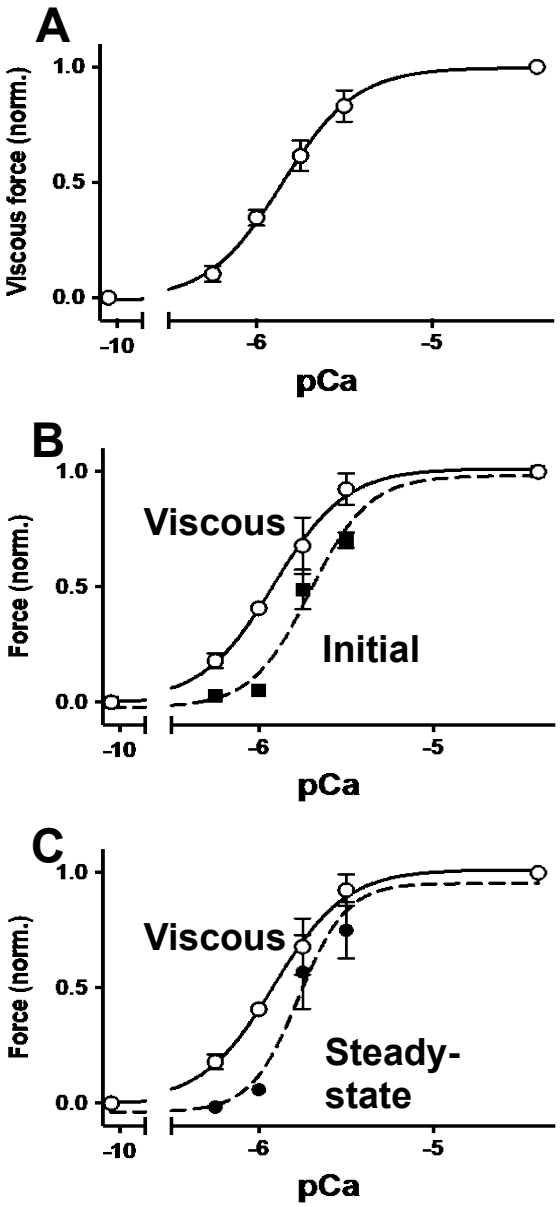


Figure 5. Ca^{2+} increased viscous stiffness (A) Viscous force (peak minus steady state) response to stretch was significantly increased at higher stretch velocity in activating/PNB conditions ($P < 0.0001$, repeated measures one-way ANOVA, open symbols females, closed symbols males). (B) Pooled data (linear abscissa scale) showing the viscous force response to stretch in activating/PNB solution was considerably higher than in relaxing/PNB solution (****$P < 0.0001$, repeated measures two-way ANOVA). (C) Pooled data showing the viscous force response to stretch in activating/PNB solution was higher in trabeculae from males than females (*$P < 0.05$, repeated measures two-way ANOVA).



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240 **Figure 6. Ca^{2+}-activation of stiffness** Pooled data showing the effect of Ca^{2+} level of the
241 viscous force response to the fastest stretch (velocity 2.25 ML/s, n = 6). Data were fit to the Hill
242 equation.

DISCUSSION

This study found that activator Ca^{2+} caused a large increase in the dynamic viscoelastic force response of cardiac muscle to stretch. The Ca^{2+}-mediated increase in viscous force was present when Ca^{2+}-activation of contraction was minimized using the inhibitor PNB. This novel effect of Ca^{2+} on cardiac muscle mechanics may be a determinant of both active and passive cardiac muscle properties. The Ca^{2+}-mediated increase in viscous component of the stretch response was present in myocardium from both males and females, and appeared greater in males.

Stress relaxation after stretch

Our experiments reveal a familiar biphasic stress response to a ramp increase in muscle length. Force increased during the stretch and reached a peak at the completion of the stretch. Following the ramp stretch, when the muscle length was held at a constant final length, the stress decayed to a steady level. The observed peak stress increased with increasing stretch velocity, even as the final length was the same for all ramp speeds. This dependence of peak stress on the rate of stretching is consistent with a nonlinear viscous component of the passive mechanical response of the muscle to stretch (5). Importantly, this magnitude of the viscous response was sensitive to stretch velocity over a physiologically relevant range of stretch velocities, suggesting relevance to the systolic and diastolic function of the beating heart.

Following a stretch, cardiac muscle is known to manifest stress-relaxation (5-8), which is consistent with a viscoelastic material response. Multiple structural elements might contribute to muscle viscous properties, including titin, actin, microtubules, intermediate filaments and the extracellular matrix (2). We found that stress-relaxation did not involve a gross change in sarcomere length, suggesting that only short-range structural changes were involved.

The observed viscous component of the stress response to stretch was not affected by PNB in relaxed muscle indicating that muscle cross-bridges did not contribute to the velocity dependency of the peak force response (Fig. 3) or to the stress relaxation. In an accompanying paper we present a theoretical analysis of the observed stress response to stretch that suggests that the passive stress relaxation proceeds over a power-law time course that is governed by the molecular mechanics of the elastic domain of titin in the I band of the sarcomere (16).

Ca^{2+} activation of cardiac muscle viscous stiffness.

Recent studies reported that electrical activation of frog skeletal increased the passive component of stiffness when active force development was prevented by the cross-bridge inhibitor PNB (1). This was suggested to result from switching of titin from an extensible spring (OFF-state) to a mechanical rectifier (ON-state) that allows shortening but has an elevated resistance to stretch (1). The titin ON state and increased muscle stiffness was hypothesized to involve cross-linking of flexible titin to relatively stiffer actin filaments (1). Electrical stimulation results in an increase of intracellular Ca^{2+} level which could trigger changes in titin properties. Indeed, previous studies have suggested Ca^{2+}-mediated stiffening of titin involves Ca^{2+} binding to the PEVK region (17) or to the immunoglobulin domain (18). Moreover, interaction between actin and titin has been proposed to increase muscle stiffness (19-21).

The current study tested the effect of activator Ca^{2+} level on cardiac muscle stiffness. Consistent with the aforementioned studies of skeletal muscle, in cardiac muscle stiffness was regulated by activator Ca^{2+} level. This phenomenon considerably expands the role of Ca^{2+} for impacting cardiac muscle mechanical properties where Ca^{2+} mediates both activation of contraction and an increase in muscle stiffness. The Ca^{2+}-mediated increased muscle viscous stiffness will likely be important in regulating both contraction and relaxation, and might play a role in development of disease.

In the presence of the cross-bridge inhibitor PNB, Ca^{2+} increased the magnitude of the viscous force response to muscle stretch by ≈ 3-fold relative to the relaxed state suggesting that cross-bridges do not play a role in mediating the Ca^{2+}-dependency of the viscous force peak. Interestingly, despite the large increase in viscous force in the activated state versus the relaxed state, the dependence of viscous force on stretch velocity was similar in both relaxing and activating conditions. This might indicate a similar mechanism underlying viscous stiffness in both active- and relaxed states.

The dependence of viscous stiffness on the level of activator Ca^{2+} followed a sigmoidal relationship similar to that observed with myofilament activation by Ca^{2+}, consistent with a coordinated effect of Ca^{2+} to both increase force development and increase viscous stiffness. Possibly, the mechanisms contributing to activation of contraction may play a role in activation of the viscous force response to stretch. The Ca^{2+}-mediated increase in cardiac muscle viscosity was greater in males than females. Potentially, this could contribute to sex-differences in systolic and diastolic behavior and warrants further study.

Limitations

The mechanism governing the Ca^{2+}-dependence of the viscoelastic properties of the muscle was not determined in this study. In the accompanying paper (16), we use a computational model to quantitatively investigate if and how Ca^{2+}-dependent binding of titin to actin (1) can account for both effects on stiffness and effects on the time-course of stress relaxation.

The observed sex-difference in the viscous force response to stretch is intriguing, but is not definitive given the small group sizes and thus requires further study.

This study used RV trabeculae; it is not clear if results are relevant to LV myocardium.

Consistent with previous studies, the myosin ATPase inhibitor PNB did not fully inhibit cardiac muscle contraction (15). Residual muscle contraction might confound the assessment of muscle stiffness. However, for all experiments, with a wide range of residual contraction level (1.5% - 26.4%), Ca^{2+} caused a similar increase in the viscous force response to stretch. This suggests that the increased viscous force response at high Ca^{2+} level was not explained by residual contraction.

Conclusion

In addition to Ca^{2+}-activation of contraction, Ca^{2+} also markedly increases the apparent viscous force response to rapid stretch of cardiac muscle. This behavior opens a new window on Ca^{2+} modulational of cardiac muscle mechanical properties that likely has important implications for understanding both systolic and diastolic properties.

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Disclosures

None

Data availability

All data available at https://github.com/beards-lab/TitinViscoelasticity/

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Conflict of interests:

None.