Effects of BDM, [Ca\textsuperscript{2+}], and temperature on the dynamic stiffness of quiescent cardiac trabeculae from rat

R. S. Kirton, A. J. Taberner, P. M. F. Nielsen, A. A. Young, and D. S. Loiselle

1Bioengineering Institute and Departments of 2Anatomy, 3Engineering Science, and 4Physiology, University of Auckland, Auckland, New Zealand; and 5Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts

Submitted 2 September 2004; accepted in final form 22 November 2004

The mechanical properties of passive cardiac tissue are important, inasmuch as they help determine the stroke volume of the heart (3) and, hence, play a major role in diastolic dysfunction observed clinically. Mechanical tests are commonly performed on isolated hearts or samples of cardiac tissue and are conducted at various temperatures and extracellular Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsubscript{o}). Recent studies (10, 11) have exploited the negative inotropic action (37) and cardioprotective properties (30) of the chemical agent 2,3-butanedione monoxime (BDM), whereas earlier studies utilized other forms of arrest. It is well established that twitch force and Ca\textsuperscript{2+} transients of activated cardiac tissues are sensitive to temperature and [Ca\textsuperscript{2+}]\textsubscript{o}. However, the influences of temperature and [Ca\textsuperscript{2+}]\textsubscript{o} on the mechanical properties of passive cardiac tissue are often overlooked. Indeed, early studies often neglected to report [Ca\textsuperscript{2+}]\textsubscript{o}, and for convenience, at room temperature. More recently, the use of BDM has further ensured passivity (10, 11); yet its effects on the mechanical properties of quiescent cardiac tissue at room temperature (20–22°C) and low [Ca\textsuperscript{2+}]\textsubscript{o} have not been explored. At 26°C and 0.5 mM [Ca\textsuperscript{2+}]\textsubscript{o}, BDM was found to have no effect on the stress vs. muscle length relationships of healthy, intact rat trabeculae (21). Similarly, the passive tension and small-amplitude sinusoidal stiffness of skinned cardiac myocytes (14) and skinned cardiac myofibrils (27) were unaffected by BDM. However, on intravascular introduction of 40 mM BDM to pig hearts in vivo, the resting diastolic fiber length was found to increase (28). Thus controversy exists within the literature regarding the presence of BDM-induced alteration of the mechanical properties of quiescent cardiac tissue.

The dynamic stiffness technique, when applied to small activated specimens of striated muscle (47, 48), has proven sufficiently sensitive to extract the rate constants of attachment and detachment of cross bridges, as well as their degree of activation. The technique has previously been used to investigate BDM-mediated changes of active tissue properties of intact rat papillary muscles (43) and rate of contracture of metabolically challenged, intact ferret papillary muscles (17), as well as the passive stiffness of skinned tissues in relaxing solutions (14, 27). However, we found no report in the literature of how BDM, temperature, and [Ca\textsuperscript{2+}]\textsubscript{o} interactively affect the amplitude and phase of dynamic stiffness of quiescent intact myocardium. The present study attempts to redress this deficit. To that end, we have examined the dynamic stiffness spectra (amplitude and phase) of intact trabeculae from the right ventricles of rat hearts, determined over a wide range of length-perturbation frequencies, at two subphysiological temperatures (20°C and 26°C) and two [Ca\textsuperscript{2+}]\textsubscript{o} (0.5 and 1.25 mM) in the presence and absence of BDM. The two [Ca\textsuperscript{2+}]\textsubscript{o} were selected to emulate the low level commonly used in the absence of BDM, the extent of spontaneous sarcomere activity decreases with increasing temperature. We caution that the measured mechanical properties of passive cardiac tissue are critically dependent on the experimental conditions under which they are measured. Experiments must be performed at sufficiently high temperatures (>25°C) to ensure a low resting concentration of intracellular Ca\textsuperscript{2+} or in the presence of an inhibitor of cross-bridge cycling. Cardiac muscle; sinusoidal length perturbation; passive mechanics; 2,3-butanedione monoxime; extracellular calcium concentration

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
during mechanical studies of passive myocardium (0.5 mM) and the physiological level for rat (1.25 mM) (4). The two temperatures where chosen to reflect room temperature (~20°C) and a just-sufficient increase (to 26°C) to allow the sarcoplasmic reticulum Ca\(^{2+}\) pump [Qo = 3.3 in this temperature range (42)] to reduce intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) to the point at which signs of sarcomere "spontaneous contractile waves" (7, 13, 41) are abolished.

**MATERIALS AND METHODS**

The University of Auckland Animal Ethics Committee approved all experiments. Wistar rats (age 59 (SD 19) days, weight 324 (SD 85) g) were killed via stunning and cervical dislocation. The thoracic cavity was opened, and the heart was quickly excised and plunged into dissection solution at 0°C to effect arrest. During Langendorff perfusion of the heart with oxygenated dissection solution, its right ventricle was opened, and a trabecula that was relatively long (2.12 (SD 0.66) mm, n = 15) and were of analytic grade. Tyrode solution. All chemicals were purchased from Sigma-Aldrich and were of analytic grade.

**Determination of stiffness spectra.** The dynamic stiffness of trabeculae was determined as described by Kirton et al. (20). Briefly, electrical stimulation was terminated, and small-amplitude sinusoidal muscle length (Lm) perturbations (~0.002 Lm), at 33 frequencies ranging from 0.2 to 100 Hz, were applied to the muscle via a piezoelectric actuator (Quensegate Technologies). The resulting change in oscillatory force was measured via a silicone beam strain gauge (model AE-801, Sensoron). The dynamic stiffness amplitude and phase were determined as the ratio of stress to strain and the physiological level for rat (1.25 mM) (4). The two groups were randomized across muscles. For a given experiment, dynamic stiffness of activated muscle. To this end, after passive muscle, in the presence and absence of BDM, with the absence of BDM and in the presence of 20 mM urea. Group 2 containing BDM had an influence on the dynamic stiffness measurements.

**Experimental protocol.** The object of the study was to determine the effects of BDM, Ca\(^{2+}\), and temperature on the dynamic stiffness of healthy quiescent trabeculae. For a given experiment, dynamic stiffness spectra were acquired at one temperature and one Ca\(^{2+}\) concentration in the presence (20 mM) and absence of BDM (+BDM and −BDM, respectively). Behavior under three different experimental conditions was tested. Group 1 (0.5 mM Ca\(^{2+}\) and 20°C) mimicked typical experimental conditions previously tested at the mechanical properties of passive myocardium: low Ca\(^{2+}\) and room temperature. Group 2 emulated physiological levels of Ca\(^{2+}\) at room temperature (20°C and 1.25 mM Ca\(^{2+}\)). For the trabeculae in group 3, [Ca\(^{2+}\)], was kept low (0.5 mM) but temperature was raised to 26°C. Within each group, the order of presentation of BDM treatments was randomized across muscles.

We examined whether the higher osmolality of the solutions containing BDM had an influence on the dynamic stiffness measurements. In six trabeculae, dynamic stiffness amplitude and phase were acquired, at 26°C in 0.5 mM Ca\(^{2+}\), in the presence (20 mM) and absence of BDM and in the presence of 20 mM urea.

Finally, we compared the dynamic stiffness spectra obtained from passive muscle, in the presence and absence of BDM, with the dynamic stiffness of activated muscle. To this end, after passive dynamic stiffness measurements of trabeculae in group 2, Ca\(^{2+}\) and BDM were washed out, via a Ca\(^{2+}\)-free solution, and a Ba\(^{2+}\) contracture was instigated (0.5 mM BaCl\(_2\), 20°C, n = 3).

**Data analysis.** To reduce the effects of the large, naturally occurring variability of stiffness between cardiac trabeculae (40), a stiffness ratio (Eq. 1) was calculated at each of the 33 measured frequencies (f).

\[
\text{stiffness ratio}(f) = \frac{\text{stiffness} + \text{BDM}(f)}{\text{stiffness} - \text{BDM}(f)}
\]

\[
\text{index XB} = \frac{\text{avg stiffness}(\text{high f})}{\text{avg stiffness}(\text{low f})}
\]

The index of cross-bridge cycling (index XB, Eq. 2), the ratio of high-frequency (in this instance, the average of 57-, 69-, 85-, and 100-Hz values) to low-frequency (in this instance, the average of 0.78-, 0.95-, 1.2-, and 1.4-Hz values) stiffness amplitude, has previously been used as an index of cross-bridge cycling (25). These indexes of cross-bridge cycling were calculated for the three interventions (groups) in the presence and absence of BDM. Because of the well-documented small increase in the amplitude of stiffness with frequency of passive muscle tissue (19, 34), index XB will always be >1. As the name implies, index XB increases with increasing cross-bridge activity (25).

**RESULTS**

It is instructive to compare the stiffness spectra of passive and active muscles. Figure 1 shows the average spectra, measured at 20°C, of nominally passive (1.25 mM Ca\(^{2+}\)) and activated (0.5 mM Ba\(^{2+}\)) trabeculae. The average stiffness amplitude spectrum, as well as the corresponding phase spectrum, of Ba\(^{2+}\)-activated trabeculae reveals frequency dependence characteristic of activated muscle (19, 33, 34).

Interestingly, the spectra of the nominally quiescent preparations show some resemblance to those observed (in the same muscles) during Ba\(^{2+}\) activation: nonlinearities and increased stiffness amplitude at high perturbation frequencies, behavior previously attributed to Ca\(^{2+}\)- or Ba\(^{2+}\)-induced cross-bridge cycling (19, 34, 38). It is striking that these spectral characteristics, generated in “quiescent” preparations, are essentially abolished by the addition of BDM. The remainder of the study focuses on the influence of reduced [Ca\(^{2+}\)]\(_o\), increased temperature, and the presence of BDM on the dynamic stiffness spectra of nominally quiescent cardiac tissue.

The dynamic stiffness amplitude and phase spectra of quiescent trabeculae were acquired for three experimental conditions (groups 1–3) to establish whether BDM alters the spectra and, if so, whether the BDM dependence is sensitive to [Ca\(^{2+}\)]\(_o\), temperature, and frequency. The average spectra for groups 1–3 are shown in Fig. 2 in the absence and presence of BDM. In the presence of 20 mM BDM, no temperature or [Ca\(^{2+}\)]\(_o\) sensitivity of the phase spectra or slopes of the stiffness amplitude spectra were observed. In contrast, when BDM was absent, phase and stiffness amplitude were noticeably sensitive to temperature and [Ca\(^{2+}\)]\(_o\).

Statistical significance of the four main effects ([Ca\(^{2+}\)]\(_o\), temperature, BDM, and frequency) and all possible interactions were analyzed by repeated-measures ANOVAs for each of the amplitude and phase data. These analyses indicated significant effects of perturbation frequency on the stiffness...
amplitude and phase spectra. In all cases, both spectra tended to increase with increasing frequency (behavior that was uninfluenced by temperature). As revealed by significant second-order interaction terms in the ANOVAs, $\text{Ca}^{2+}$ and BDM affected both spectra. Thus, raising $[\text{Ca}^{2+}]_o$ from 0.5 to 1.25 mM in the absence of BDM caused a disproportionate increase in stiffness amplitude at higher perturbation frequencies (Fig. 2A). It also increased the phase, albeit disproportionately at lower frequencies (Fig. 2B). BDM had the striking effect of rendering indistinguishable (and quasi-linear) the phase spectra of all three groups (Fig. 2D). Similarly, BDM linearized the stiffness amplitude spectra, independent of $\text{Ca}^{2+}$ and temperature (Fig. 2C).

The single, somewhat anomalous, result in Fig. 2 is the higher stiffness amplitude of group 2 (1.25 mM $\text{Ca}^{2+}$ and 20°C) in the presence of BDM. We reasoned that the muscles assigned to group 2 might have been inherently stiffer, reflecting a naturally occurring variation of stiffness among trabeculae as noted by Stuyvers et al. (40). To reduce the effect of variation of inherent stiffness, we calculated a normalized stiffness ratio ($\text{BDM}/(\text{BDM})$, Eq. 1) for each trabecula. The ratios for the three groups are plotted in Fig. 3. This normalization procedure abolished the previous apparent effect of $\text{Ca}^{2+}$ on stiffness amplitude. Interestingly, under all three superfusion condi-

![Fig. 1. Average (mean ± SE) dynamic stiffness amplitude (A) and phase (B) spectra for cardiac trabeculae (20°C, $n = 3$) when quiescent and BDM free (dark gray dashed line), when quiescent with the addition of 20 mM BDM (light gray line), and when activated (light gray line). Lines represent linear interpolation of acquired stiffness and phase data.](image1)

![Fig. 2. Average (mean ± SE) dynamic stiffness amplitude (A and C) and phase (B and D) spectra of quiescent trabeculae acquired in the absence (A and B) and presence (C and D) of 20 mM BDM. Results are from 3 experimental protocols: 0.5 mM $\text{Ca}^{2+}$ and 20°C ($n = 7$, dark gray dashed line, group 1), 1.25 mM $\text{Ca}^{2+}$ and 20°C ($n = 3$, light gray line, group 2), and 0.5 mM $\text{Ca}^{2+}$ and 26°C ($n = 5$, black line, group 3).](image2)
tions, BDM decreased stiffness amplitude above ~9 Hz (stiffness ratios > 1) and increased it below ~9 Hz (stiffness ratio < 1).

To interrogate our data more completely, we utilized the index of cross-bridge cycling (index XB, Eq. 2) devised by Leijendekker et al. (25) (Fig. 4). In the presence of BDM, index XB did not differ among groups. In contrast, index XB was significantly greater in the absence of BDM but only at the lower temperature.

It may be argued that the BDM-induced alterations of dynamic stiffness amplitude and phase (Figs. 2 and 3) arose from the 20 mosM higher osmolarities of the superfusates in the presence of BDM. This possibility was examined by comparing the spectra acquired in the presence of BDM, in the absence of BDM, and with urea (20 mM) at 26°C and 0.5 mM Ca2+. If the observed dynamic stiffness and phase changes were results of the higher osmolarity, then BDM and urea would be expected to effect similar changes. Repeated-measures ANOVA, followed by mutually orthogonal contrasts, showed no significant differences of dynamic stiffness between the −BDM and urea groups (P = 0.42, n = 6), although both were significantly different from the stiffness measured in the +BDM group (P < 0.0001, n = 6). Thus the 20 mosM higher osmolarity of the BDM-containing solution was not responsible for the observed changes in stiffness.

**DISCUSSION**

To reveal the mechanical behavior of passive cardiac muscle, it is first necessary to eliminate any contribution to resting force by actomyosin cross bridges. This has traditionally been achieved by the combined use of low [Ca2+]o and low temperature. Common experimental conditions are 0.5 mM Ca2+ and room temperature. However, when right ventricular trabeculae from rat were subjected to such experimental conditions, spontaneous, asynchronized “waves” of contractile movement, commonly called “writhing,” are reported (7, 13, 21, 41). In trabeculae that were deemed to be viable, raising the temperature to 25°C significantly reduced the degree of writhing (7, 21). This observation is consistent with the notion that the sarcoplasmic reticulum Ca2+-ATPase fails to cope with spontaneous Ca2+ leak under conditions where its high Q10 fenders it ineffective. The concomitant reduction in rate of Na+/Ca2+ exchange at lower temperatures means that both principal mechanisms for removal of Ca2+ from the cytoplasm are inhibited at low temperatures (2). Hence, it seems likely that, for any given [Ca2+]o, [Ca2+], (and, hence, the degree of cross-bridge cycling and resting tension) is higher at lower temperatures in nominally quiescent cardiac muscle tissue. Such phenomena have been reported, without elaboration, during dynamic stiffness measurements of intact, quiescent kitten papillary muscles, in which increased temperature resulted in decreased dynamic stiffness (34).

We have investigated these effects during experiments using right ventricular trabeculae from rat. This species is characterized by a high basal rate of cardiac metabolism (13) and a high occurrence of spontaneous mechanical oscillations of quiescent cardiac tissues (22, 24), behaviors consistent with an inherently “leaky” sarcoplasmic reticulum and, by implication, an inherently high degree of cross-bridge cycling during “rest.” It is thus an ideal species in which to examine the effects, on the mechanical properties of passive cardiac muscle, of temperature and Ca2+, in combination with BDM, an inhibitor of cross-bridge activity. It must be emphasized that such investigation must be performed in cardiac tissue that is known to be healthy (i.e., to respond to electrical stimulation), inasmuch as recent studies have demonstrated that the stress vs. muscle length relationships (21) and dynamic stiffness (20) of passive cardiac tissue are significantly altered on loss of tissue viability.

We used three indexes to examine the effect of BDM on passive myocardium: 1) the spectra (amplitude and phase) of dynamic stiffness as functions of the frequency of perturbation of muscle length, 2) the ratio of stiffness amplitude spectra in the presence and absence of BDM (Eq. 1), and 3) an index of cross-bridge cycling defined as the ratio of high-frequency stiffness to low-frequency stiffness (Eq. 2). As shown in Fig. 1, the stiffness spectra are sufficiently sensitive to distinguish quiescent tissues from those that are only partially activated. Figure 2 demonstrates that sensitivity is sufficient to reveal the differential effects of various superperfusion conditions on quiescent tissues. In particular, BDM is seen to “linearize” otherwise nonlinear stiffness spectra while convincingly removing any effects of temperature or Ca2+ on the phase spectra (Fig. 2D), which is consistent with its reputed ability to inhibit cross-bridge activity in viable tissue. The normalization procedure underlying the data of Fig. 3 provides a method of “correcting” for the inherent variability of stiffness that characterizes cardiac trabeculae (40) and that had, in this case, led to an apparent effect of extracellular Ca2+ in the presence of BDM.

The effectiveness of BDM as an inhibitor of cross-bridge cycling is probably best revealed by the data of Fig. 4, where a putative increase in [Ca2+], at 20°C (a temperature that greatly slows the sarcoplasmic reticulum Ca2+-pump) had no effect on index XB in the presence of BDM. Figure 4 shows that an actin-myosin inhibitor, such as BDM, is crucial for correctly determining the mechanical properties of passive cardiac tissue at room temperature. This requirement is substantially alleviated if experiments are conducted at >25–26°C.

These data appear to be consistent with BDM having an inhibitory action on “residual” cross-bridge activity in nominally quiescent cardiac muscle tissue. However, there remains the unexpected observation (Fig. 3) that, independent of temperature or [Ca2+]o, BDM increased dynamic stiffness ampli-
tude at low perturbation frequencies (<9 Hz) and decreased it at higher frequencies. Possible candidates to which this behavior might be attributed include 1) titin, 2) weakly bound cross bridges, and 3) active cross bridges.

**Titin.** Recently, the majority of passive tension developed by cardiac tissue at low sarcomere length extensions has been attributed to the intracellular elastic protein titin (14, 44). Ca\(^{2+}\)-dependent titin-actin interactions (15, 26, 41), as well as Ca\(^{2+}\)-sensitive molecular domains within titin (12, 23), are reported to modulate passive stiffness. Thus the possibility exists that BDM modifies these interactions and results in the frequency-dependent alterations of dynamic stiffness observed in Fig. 3. BDM was shown to have no effect on the stiffness or passive tension of skinned rat myocytes (14) or skinned rabbit (26) or rat (27) cardiac myofibrils in relaxing (low-Ca\(^{2+}\)) solutions. The stiffness associated with Ca\(^{2+}\)-dependent titin-actin interactions was shown to be small for intact and skinned rat trabeculae (41). The stiffness changes associated with the Ca\(^{2+}\)-responsiveness of the PEVK segment of titin was also shown to be small for specimens from skeletal muscle (23) and cow atria (12) and were absent in rat trabeculae (12). These Ca\(^{2+}\) sensitivities were shown to be insensitive to 30 mM BDM.

Yamasaki et al. (45) demonstrated that protein kinase A phosphorylates titin in skinned rat myocytes, thereby lowering passive tension generated during physiological levels of stretch. Hence, if BDM acts on titin as a phosphatase, it would be expected to increase passive tension and, hence, stiffness of cardiac tissue. However, no such effect has been reported in high (12) or low (14, 27) Ca\(^{2+}\) in skinned cardiac preparations. Furthermore, even if a BDM-induced increase in the passive tension-length relation had been observed in intact cardiac trabeculae [which is unlikely, because Kirton et al. (21) found no increase], this would more likely have caused an increase of stiffness at all frequencies [as observed during the formation of rigor cross bridges (20)] than the frequency dependence shown in Fig. 3.

Collectively, these reports imply that the observed BDM-induced reduction of low-frequency stiffness cannot be fully explained by Ca\(^{2+}\)-sensitive titin interactions or by BDM-induced dephosphorylation of titin.

**Weakly bound cross bridges.** Controversy exists over the presence of weakly bound cross bridges in striated muscle. It has been reported that weakly bound cross bridges contribute to stiffness and tension of skeletal muscle (16, 35). Conversely, no evidence was found for the existence of weakly bound cross bridges in intact frog muscle (1), intact cardiac trabeculae (8), or skinned rabbit or rat cardiac myofibrils (26, 27).

Nevertheless, from the results of mechanical (30, 47) and paramagnetic (46) studies, it is hypothesized that BDM reduces the population of force-bearing and increases the population of weakly bound, cross bridges. The present study found that stiffness and phase spectra were sensitive to temperature in the absence of BDM but insensitive in its presence (Fig. 2). Therefore, it is unlikely that the BDM-induced increase in low-frequency (<9 Hz) stiffness (stiffness ratio > 1; Fig. 3) can be attributed to weakly bound cross bridges.

**Force-bearing cross bridges.** Further controversy exists over the presence of force-bearing cross bridges in quiescent cardiac tissue. Thus the presence (28) and absence (18) of significant numbers of active cross bridges during the diastolic interval of whole hearts in vivo have been reported. Similar controversy exists with respect to cardiac tissue in vitro. Isolated intact myocardial cells from rat were reported to have modest cross-bridge activity during diastole (39), as were segments of rabbit papillary muscle (29), whereas during the diastolic interval in rat trabeculae, an absence of active cross bridges was reported (8). The mechanism for the effect of BDM on the frequency response of passive myocardium remains unknown. One hypothesis is that, in the absence of BDM, cross bridges may cycle asynchronously but become synchronized (with nonzero phase angle) when perturbed at low frequency. This synchronization induces a low-level minimum in the dynamic stiffness spectra at low frequencies (Fig. 1) that is comparable to that seen in maximally activated striated muscle (19, 34). The BDM inhibition of these spontaneous cross bridges renders the tissue passive (i.e., no cross-bridge activity) and, counterintuitively, increases the low-frequency amplitude stiffness via abolition of the minimum. This behavior has not previously been reported.

**limitations.** A limitation of this study is that sarcomere length was not acquired for all preparations. Differences in initial sarcomere length (1.97 ± 0.07 μm for the 12 preparations in which it was recorded) might thus explain the increased stiffness of trabeculae in group 2 (Fig. 2C). Care was therefore taken to normalize dynamic stiffness by treating each preparation as its own control. This was achieved for the data of Fig. 3 by dividing the stiffness in the presence of BDM by that in its absence within each muscle and at each frequency. Likewise, for the data of Fig. 4, the index of cross-bridge cycling was normalized by dividing the high-frequency stiffness by the low-frequency stiffness on a per-muscle basis. Finally, all trabeculae showed consistent phase spectra in the presence of BDM (Fig. 2D). Hence, we consider that this methodological limitation does not affect any of our conclusions.

In conclusion, at room temperature (20°C), viable trabeculae contain enough asynchronously cycling force-bearing cross bridges to cause frequency-dependent variations in the dynamic stiffness amplitude and phase spectra. This putative cross-bridge activity can be reduced by an increase in the experimental temperature to 26°C and completely inhibited by addition of 20 mM BDM. Therefore, when mechanical experiments are conducted on passive cardiac tissue, it is critical to perform them at >25°C or in the presence of an inhibitor of cross-bridge cycling. The lower values of dynamic stiffness at low frequencies in the absence of BDM deserve further study.

**GRANTS.** Generous financial support for this study was provided by the Royal Society of New Zealand (Marsden Fund), Foundation for Research Science and Technology Contract MASS0101, and the Health Research Council of New Zealand.

**REFERENCES.**


