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Preload-induced changes in isometric tension and $[Ca^{2+}]_i$ in rat myocardium

Research Article

Oleg Lookin*, Yuri Protsenko

Laboratory of biological motility, Institute of Immunology and Physiology, Ural Branch of Russian Academy of Sciences, 620049 Ekaterinburg, Russia

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Abstract: Preload-induced changes of active tension and $[Ca^{2+}]i$ are "dissociated" in mammalian myocardium. This study aimed to describe the distinct effects of preload at low and physiological $[Ca^{2+}]_o$. Rat RV papillary muscles were studied in isometric conditions at 25°C and 0.33 Hz at 1 mM (hypo-Ca group) and 2.5 mM $[Ca^{2+}]_o$ (normal-Ca group). $[Ca^{2+}]_i$ was monitored with fura-2/AM. Increase of preload caused a rise of active tension in hypo-Ca and normal-Ca groups whereas peak fluorescence rose significantly only at low $[Ca^{2+}]_o$. End-diastolic tension, end-diastolic level of fluorescence, time-to-peak tension, but not time-to-peak of Ca^{2+} transient, progressively increased with preload. Mechanical relaxation decelerated with preload while Ca^{2+} transient decay time decreased in the initial phase and increased in the late phase, resulting in a prominent "bump" configuration. The "bump" was assessed as a ratio of its area to the fluorescence trace area. It was a new finding that the preload-induced rise of this ratio was twice as large in hypo-Ca. Our results indicate that preload-induced changes in active tension and $[Ca^{2+}]_i$ are "dissociated" in rat myocardium, with relatively higher expression at low $[Ca^{2+}]_o$. Ca-dependence of Ca-TnC association/dissociation kinetics is thought to be a main contributor to these preload-induced effects.

Keywords: *Muscle stretch • Calcium (Ca^{2+}) transient • Relaxation*

1. Introduction

Myocardial contractility is regulated *via* force-length relation (Frank-Starling Law for whole heart) that links a maximal isometric force with a degree of muscle stretch or preload [1]. It is well established that an increase of preload in the physiological range results in an increase of peak isometric force or afterload shortening, a rise of maximal rates of force development and relaxation as well as time-to-peak tension and relaxation time. The primary mechanism of force-length relation is the preload-dependence of sarcomere filament overlap which dictates the total number of sites available for interaction of myosin filament with actin [2-4]. This indirectly results in augmentation of troponin C (TnC) affinity to Ca^{2+} in mammalians [5-7].

Preload-dependent changes in calcium handling in cardiomyocytes are more complex and controversial. No preload-dependence of resting $[Ca^{2+}]_i$ level has been shown in ferret papillary muscle [8], mouse right ventricular trabeculae [9], and stimulated or quiescent Guinea-pig, rat and mouse cardiomyocytes [10-14]. On the other hand, preload caused a rise in resting calcium level in stimulated Guinea-pig right ventricular trabeculae [15] or a small significant decrease of that level in rat ventricular trabeculae [16,17].

The amount of Ca^{2+} entering to or extruding from cytosol during a twitch, and the kinetic properties of the process, also depend on the preload. The higher preload the larger amplitude of Ca^{2+} transient has been observed in mouse [9], cat [18], rat [16,17,19] and rabbit [20,21] ventricular tissue. This preload-dependent increase of Ca^{2+} transient amplitude was smaller at higher $[Ca^{2+}]_o$ [18]. In contrast, no preload-dependent changes in Ca^{2+} transient amplitude have been shown in ferret [22], rat [18,23] and human myocardium [24] while marked increase of peak isometric force was observed in all the cases. The relative invariance of Ca^{2+} transient amplitude during the action of force-length relation was interpreted as a "downstream"

* *E-mail: o.lookin@iip.uran.ru*

mechanism in regulation of force generation and calcium handling in myocardium [25].

It has been shown that in ferret, cat and rat myocardium [18,22] and isolated Guinea-pig and rat myocytes [12,26,27] the Ca^{2+} transient declines faster at high preload. Superimposing of Ca^{2+} transients obtained in rat cardiac muscle at low and high preload revealed their crossing on approximately half of the transient amplitude [16,17,23]. With an increase of preload, the faster Ca^{2+} transient decline was observed in the initial phase while the decline decelerated in the late phase [16,23]. The rate of the Ca^{2+} transient decline in the initial phase was strengthened by increasing of $[Ca^{2+}]_o$ [22,28]. However, no evidence of preload effect on the rate or total duration of Ca^{2+} transient decline was found in rabbit trabeculae [21] and Guinea-pig ventricular myocytes [29].

This highlights a large pool of data, obtained in experiments on warm-blooded animals, that demonstrate that preload induces changes in both active force and Ca^{2+} transient but not in the same manner and/or degree. In this study we aimed to investigate in detail, the effects of preload on both isometric twitch and free intracellular Ca^{2+} in rat cardiac muscles. We used physiological and low concentration of external calcium to find whether the total amount of Ca^{2+} in cytosol may play a role in the preload-dependent changes in force and Ca^{2+} transient. The main purpose was to estimate the degree of "dissociation" between the preload-induced effects, and thus to clarify the functional interplay between muscle length (preload), free cytosolic Ca^{2+} and force development in rat myocardium.

2. Experimental Procedures

2.1 Animals

Animals were treated in accordance with the policies and regulations approved by the Institute of Immunology and Physiology of Ural Branch of RAS Animal Welfare Committee.

2.2 Isolation of papillary muscle

White out-bred rats (male and female, aged 5 months, weighted ~250 g) were stunned by a blow to the head and were killed by cervical dislocation. The heart was quickly excised and placed in modified Krebs solution containing (in mM): NaCl 118.5; KCl 4.2; $MgSO_4$ 1.2; $CaCl_2$ 1; glucose 11.1, buffered with $NaHCO_3$ and KH_2PO_4 at room temperature to maintain pH = 7.35. 2,3-butanedione monoxime (BDM, 30 mM) was added to the modified solution to prevent tissue damage during dissection. The right ventricle was opened and thin well-shaped papillary muscles were carefully dissected from the ventricle. The muscle was attached to a force transducer and a length servomotor and placed in an experimental chamber, where it was continuously washed by the saline solution, bubbled with 95% O_2+5% CO_2 at 25°C, and paced at 0.33 Hz with 1.5 threshold amplitude rectangular electrical pulses. After an equilibration period in modified Krebs solution (see composition above), we used 1 mM or 2.5 mM of $CaCl_2$ to study preload-dependent changes of active force and calcium transient at different Ca-activation. Only one $CaCl_2$ concentration was used for any particular muscle (n=12 for 1 mM $CaCl_2$, n=15 for 2.5 mM $CaCl_2$) because of fluorescent dye photobleaching during the experimental protocol.

2.3 Dye loading

Fura-2/AM was used to monitor free intracellular calcium. The ratiometric dye was chosen to avoid possible movement artifacts. Our tests showed that neither muscle movement during contraction nor preload-dependent change of the muscle surface affected measured fluorescence.

50 μM stock solution was prepared from 1 mg fura-2/AM powder dissolved in 1 ml dimethyl sulphoxide (DMSO) and stored at –20°C until the beginning of an experiment. The loading solution contained 5 μM fura-2 and 5% w/v Pluronic F-127 to promote better loading, the final concentration of DMSO in the loading solution did not exceed 0.5%. The loading procedure lasted 1.5 hours at room temperature (22-23°C) with a low pacing rate (0.2 Hz) in continuously flowing and bubbled loading solution. The low temperature and pacing frequency were chosen to diminish degradation of dye during the loading. After the loading period, solution was changed to modified Krebs solution and muscle was equilibrated for at least 60 minutes, contracting at 25°C and 0.33 Hz. The fluorescence measured after the loading procedure was suitable for measurements in ~90% of preparations.

2.4 Isometric twitch and fluorescent measurements

The simultaneous measurement of both muscle force and intensity of fura-2 fluorescence was carried out with Muscle Research System (Scientific Instruments GmbH, Heidelberg, Germany). The configuration and properties of the system allowed excitation at 340/380 nm wavelengths and measurement of fluorescence at 510 nm wavelength every 2.5 ms. To prevent dye photobleaching and diminish phototoxicity the fluorescence was collected periodically during short intervals (<30 s). Force and fluorescence were sampled

at 10 kHz by analog-to-digital (A/D) and D/A data converter PCI-1716S (AdLink Technology Inc., Taiwan) using self-made software ran under HyperKernel real-time environment (Arc Systems Ltd., Japan) integrated to Windows XP. Data were processed and analyzed off-line using self-made software.

2.5 Experimental Protocols

After the equilibration period a muscle was released to slack length (L_0) and then sequentially stretched (with step of 2-5% of L_0) and allowed to reach the steady state level at each new preload. Isometric twitch and calcium transient were measured simultaneously before the next stretch. The stretch was stopped when a large increase of end-diastolic tension with no change or decrease in peak active tension was reached, allowing measurement of the length of maximal active isometric tension (L_{MAX}). The two groups of muscles with different $CaCl_2$ concentration in washing solution were studied separately, instead of consecutive change of the concentration for the same muscle. The reason was to avoid the possible contribution of dye photobleaching during long measurement at different $CaCl_2$ concentrations. All the studied preparations were subjected to the same range of preloads. Only in few cases we did not tested large preloads (>125% of L_0), because of steep rise of passive tension. In most of preparations, however, the tested preloads were ranged from 100 to >130% of L_0.

2.6 Data analysis

Isometric twitches and calcium transients obtained in steady-state conditions at each preload were averaged by 10 consecutive cycles. Nominal muscle stress or tension (expressed as P) was calculated as ratio of force and cross-sectional area measured at slack length, assuming elliptical shape of that section. Conversion of fluorescence to calcium concentration was not performed because we were interested in relative preload-induced changes of Ca^{2+} transient. Prior to analysis all the tested preloads were divided into 4 ranges: <105% L_0 (assumed as no stretch), 105-115% L_0, 115-125% L_0, 125-135% L_0. Preload-dependent effects were estimated for active and passive tension, maximal rates of the tension development/relaxation, time-to-peak tension, time of the relaxation (estimated at the level of 80%, 70%, 60%, 50%, 40%, 30% and 20% of peak tension, T_{80}, T_{70}, …, T_{20}). Similar parameters of fluorescence (expressed as F) were estimated. The value of a measured parameter at any preload was expressed as a % of the value obtained at slack length L_0. Groups of muscles at physiological (2.5 mM, normal-Ca) and low (1 mM, hypo-Ca) $[Ca^{2+}]_o$ concentrations in saline solution were compared.

2.7 Chemicals and reagents

All chemicals used for saline solution preparation and BDM were purchased from Sigma-Aldrich (USA). Fura-2/AM was purchased from Fluka Biochemika (Switzerland).

2.8 Statistics

Data were statistically evaluated using one-way (single factor) ANOVA, $P<0.05$. All data are presented as means ± S.E.M.

3. Results

3.1 Effect of preload on peak/diastolic tension and fluorescence

Both in normal-Ca and hypo-Ca groups the active isometric tension increased with muscle stretch over the range of 100% to 135% L_0 (Figure 1A). This increase was smaller in hypo-Ca group (2.2±0.3 times per each 10% of length increase) than in normal-Ca group (2.5±0.1 times). In contrast, the progressive preload-induced increase in difference between peak and end-diastolic levels of fluorescence (that is, amplitude of fluorescence signal) was shown in hypo-Ca group only (Figure 1B, grey symbols). In normal-Ca group this amplitude did not differ significantly from the value at L_0 with muscle stretch up to 125% L_0, but an increase was detected at preloads >125% L_0 (Figure 1B, black symbols). As a result, when a muscle was stretched to 105-115% or 115-125% L_0, a significantly higher increase in the amplitude of fluorescence was observed in low $[Ca^{2+}]_o$ solution, compared with normal-Ca group.

The passive (end-diastolic) tension rose with preload non-linearly in hypo-Ca and normal-Ca groups with similar gain of 4.3±0.4 and 4.7±0.7 times, respectively, per each stretch amounted to 10% L_0 (Figure 1C). No significant differences between the two groups were detected, whatever preload range was used. The end-diastolic level of fluorescence, similarly to the passive tension, was lowest at slack length L_0, and progressively increased with preload. This took place in both hypo-Ca and normal-Ca groups with more effect seen in normal-Ca solution (Figure 1D). This resulted in a significant difference from hypo-Ca group at preload ranges of 105-115% and 125-135% L_0 ($P<0.05$), but not in the range of 115-125% L_0.

Therefore, the “dissociation” between preload-induced increase of active tension and preload-independence of amplitude of Ca^{2+} transient was found at normal $[Ca^{2+}]_o$ levels, whereas in hypo-Ca solution the increase in active tension, evoked by muscle stretch, was accompanied by the rise of free cytosolic Ca^{2+}

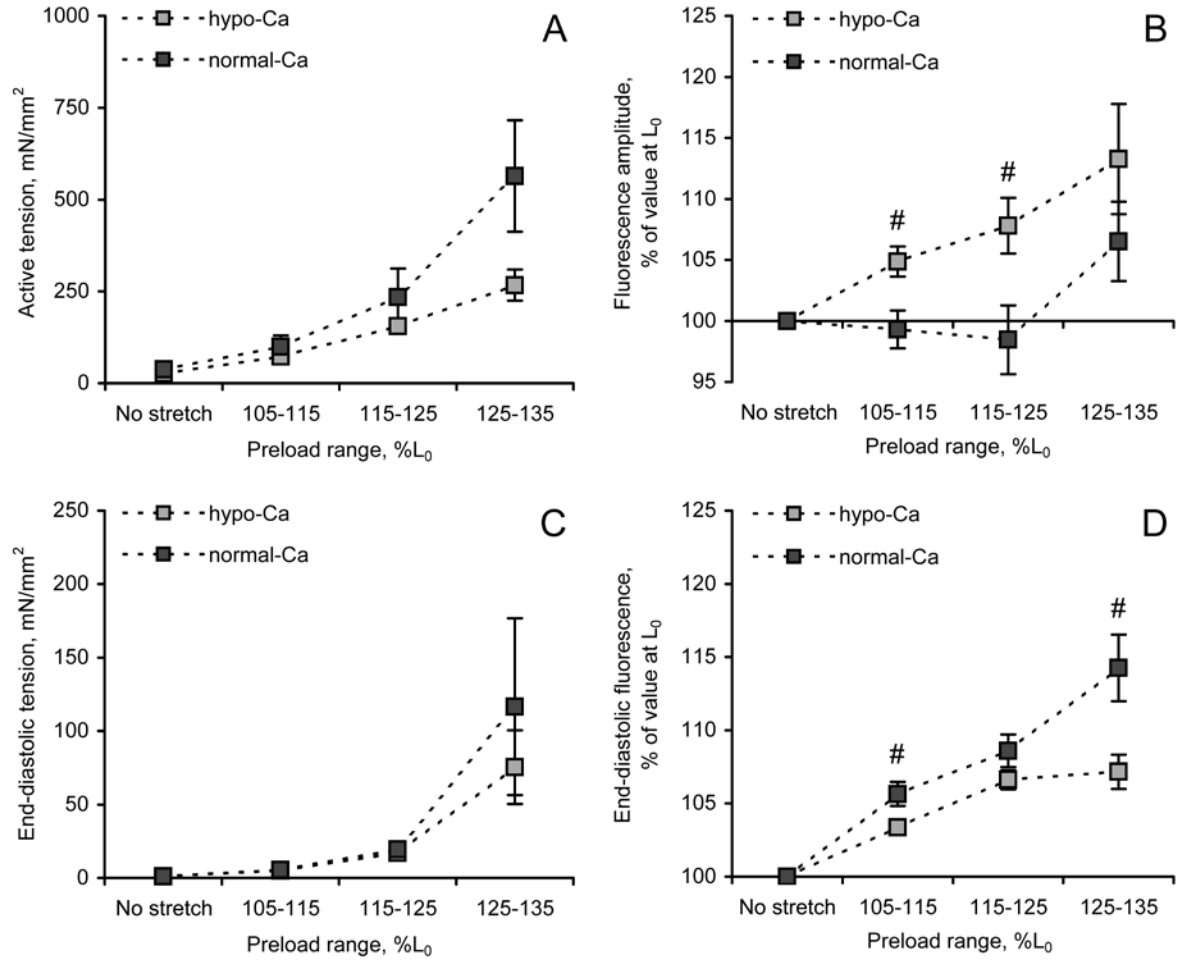

Figure 1. Preload-dependent changes in peak/end-diastolic values of tension and fura-2 fluorescence. Preload-dependent changes in active and end-diastolic tension (A and C, respectively) and amplitude and end-diastolic level of fura-2 fluorescence (B and D, respectively) are shown. Tension is expressed in absolute values, fluorescence is expressed in % of the corresponding value measured at L_0. Data are obtained for hypo-Ca group (light grey symbols) and normal-Ca group (dark grey symbols). # - significant difference between hypo-Ca and normal-Ca groups for the given preload range; $P<0.05$.

concentration. In contrast, similar preload effects on passive tension and resting $[Ca^{2+}]_i$ level were shown.

3.2 Effect of preload on maximal rates of rise/decline of tension and fluorescence

Maximal rates of isometric tension development $(dP/dt)_{MAX}$ and relaxation $(dP/dt)_{MIN}$ were significantly higher at any muscle length than at L_0, in both hypo-Ca and normal-Ca groups, with higher preload-dependent increase of the rate in normal-Ca group (Figure 2A and 2C). This increase was insignificantly higher for $(dP/dt)_{MAX}$, compared with $(dP/dt)_{MIN}$, in both hypo-Ca and normal-Ca groups. Stretch above 125% L_0 did not lead to any further significant change of $(dP/dt)_{MAX}$ or $(dP/dt)_{MIN}$ respective to their values at preloads <125% L_0. Moreover, in the normal-Ca group the relative increase in $(dP/dt)_{MAX}$ and $(dP/dt)_{MIN}$ for preloads above 125% L_0 was not significantly different from that increase obtained for preloads ranged in 105-115% L_0.

Maximal rates of both rising phase $(dF/dt)_{MAX}$ and decline $(dF/dt)_{MIN}$ of fluorescence were higher at any preload than at an un-stretched state and progressively increased with preload in hypo-Ca solution (Figure 2B and 2D, light grey bars). In normal-Ca group the mean value of $(dF/dt)_{MAX}$, obtained for preload ranges of 105-115% and 115-125% L_0, was not different from value at L_0, but was significantly higher for preloads >125 L_0 (Figure 2B, dark grey bars). Similarly, $(dF/dt)_{MIN}$ showed no difference from value at L_0 for preloads above 115% L_0 or even decreased relative to this value at preload ranged from 105 to 115% L_0 (Figure 2D, dark grey bars). These different effects resulted in maximal rates of fluorescence rise and decline that were significantly higher in hypo-Ca group compared

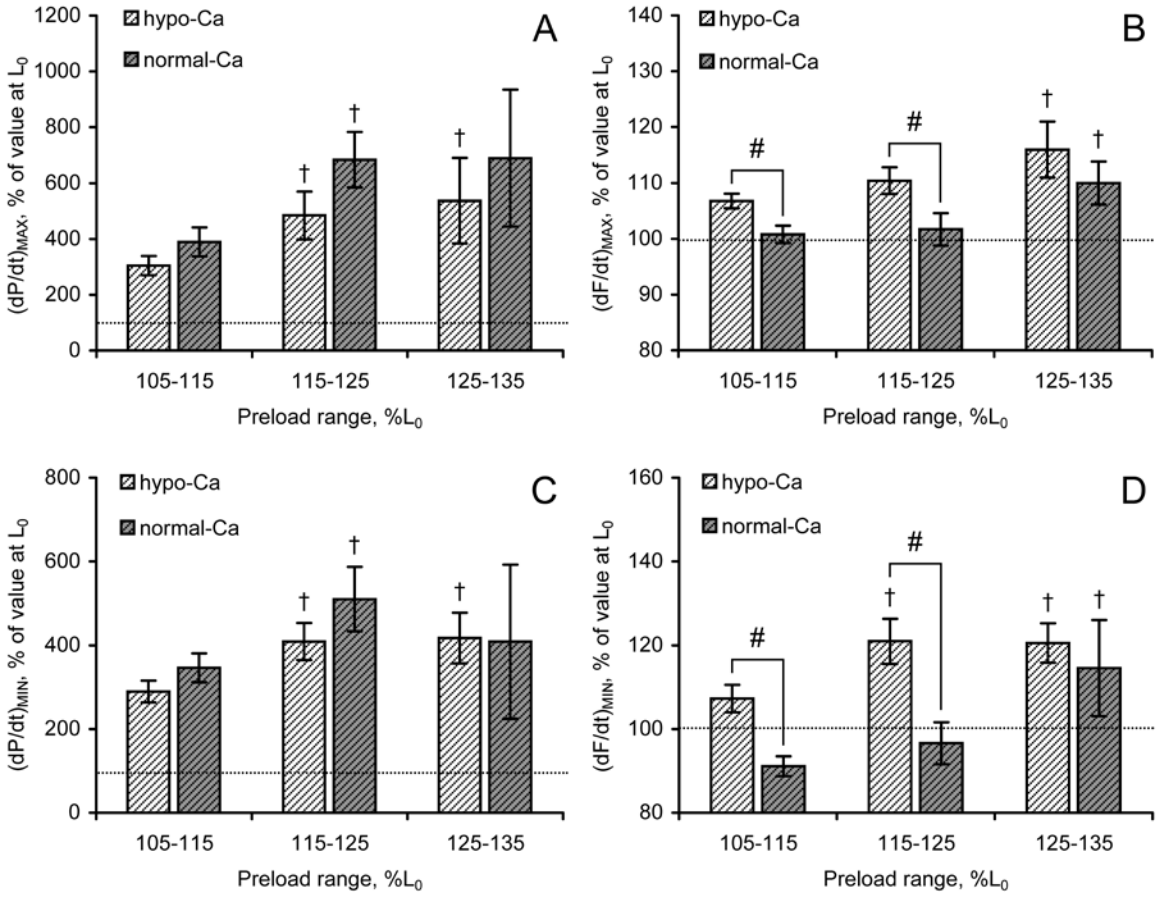

Figure 2. Preload-dependent changes in maximal rates of active tension development and relaxation and fura-2 fluorescence rise and decay. Preload-dependent changes (expressed as % of value at L_0) in maximal rates of active tension development (A, $(dP/dt)_{MAX}$) and relaxation (C, $(dP/dt)_{MIN}$) and maximal rates of fura-2 fluorescence rise (B, $(dF/dt)_{MAX}$) and decay (D, $(dF/dt)_{MIN}$) are shown. Data are obtained for hypo-Ca group (light grey bars) and normal-Ca group (dark grey bars). # - significant difference between hypo-Ca and normal-Ca groups for the given preload range; † - significant difference from preload range of 105-115% of L_0 within the same group; $P<0.05$. Dotted horizontal lines indicate 100% level (value at L_0).

with normal-Ca, for preloads in 105-115% and 115-125% L_0 ($P<0.05$).

Unlike the maximal rates of tension development and relaxation, the rates normalized to peak active tension ($(dP/dt)_{MAX}/P_{MAX}$ and $(dP/dt)_{MIN}/P_{MAX}$, respectively) were significantly less than at L_0 in both groups (Figure 3A and 3C). The relative preload-dependent decrease of $(dP/dt)_{MIN}/P_{MAX}$ was higher compared with $(dP/dt)_{MAX}/P_{MAX}$. No significant difference in both parameters was found between hypo-Ca and normal-Ca groups, regardless of the preload range.

The maximal rate of the rising phase of fluorescence signal, normalized to its amplitude, $(dF/dt)_{MAX}/F_{MAX}$, increased with preload and was higher compared with L_0, with no significant difference between hypo-Ca and normal-Ca groups (Figure 3B). The normalized maximal rate of fluorescence decline $(dF/dt)_{MIN}/F_{MAX}$ behaved similarly in hypo-Ca group (Figure 3D, light grey bars). In the normal-Ca group, in contrast, the value of $(dF/dt)_{MIN}/F_{MAX}$ did not differ from the value at L_0 for preload >115% L_0, and was significantly smaller at preload ranged in 105-115% L_0 ($P<0.05$). This led to significantly higher values of $(dF/dt)_{MIN}/F_{MAX}$ in hypo-Ca group than in normal-Ca group for preloads of 105-115% and 115-125% L_0 (Figure 3D).

The normalized rates are dependent on absolute values of peak active tension/fluorescence, therefore the data presented in Figure 3 interrelated with those in Figures 1 and 2. For example, concomitant preload-induced *increase* of both active tension and maximal rates of tension development/relaxation resulted in preload-induced *decrease* of normalized maximal rates (that is expressed as $(dP/dt)/P_{MAX}$), indicating that the preload-induced increase was higher for peak tension. Preload-dependent behavior of normalized maximal rate of fluorescence decline showed similarity with the rates,

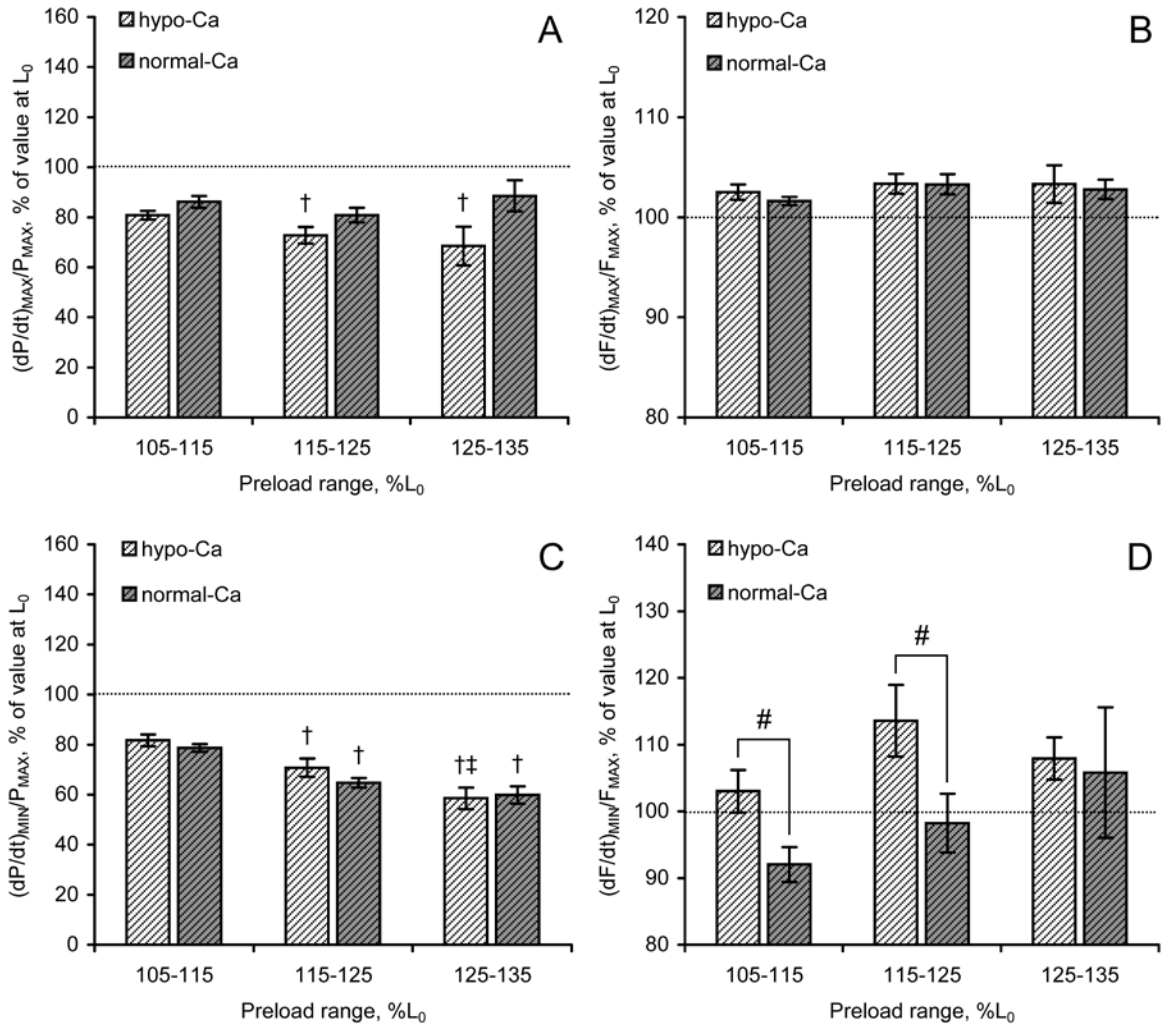

Figure 3. Preload-dependent changes in normalized maximal rates of active tension development and relaxation and fura-2 fluorescence rise and decay. Preload-dependent changes (expressed as % of value at L_0) in maximal rates of active tension development (A, $(dP/dt)_{MAX}/P_{MAX}$) and relaxation (C, $(dP/dt)_{MIN}/P_{MAX}$) normalized to amplitude of the tension, and maximal rates of fura-2 fluorescence rise (B, $(dF/dt)_{MAX}/F_{MAX}$) and decay (D, $(dF/dt)_{MIN}/F_{MAX}$) normalized to amplitude of the fluorescence are shown. Data are obtained for hypo-Ca group (light grey bars) and normal-Ca group (dark grey bars). # - significant difference between hypo-Ca and normal-Ca groups for the given preload range; † - significant difference from preload range of 105-115% of L_0 within the same group; ‡ - significant difference from preload range of 115-125% of L_0 within the same group; $P<0.05$. Dotted horizontal lines indicate 100% level (value at L_0).

calculated without normalizing (compare Figure 2D and 3D, light grey bars). In contrast, the normalized maximal rate of fluorescence rise was altered relative to the rate without normalizing, resulted in a disappearance of the significant difference between hypo-Ca and normal-Ca groups (compare Figure 2B and 3B).

In general, maximal rates of tension rise and relaxation revealed similar preload-dependent behavior as those for fluorescence. However, normalized maximal rates of tension development/relaxation decreased with preload increase, whereas normalized maximal rates of Ca^{2+} transient rise and decline both revealed preload-induced increase or, at least, were higher compared with values at L_0.

3.3 Effect of preload on time-to-peak tension and fluorescence

Time-to-peak tension was higher in stretched muscle (regardless of the preload value) than at L_0, similarly in both hypo-Ca and normal-Ca groups (Figure 4A). In both groups time-to-peak tension increased significantly for preloads ≤125% L_0. The parameter obtained for preloads ranged in 125-135% L_0 showed no further preload-induced increase in hypo-Ca group, and even revealed decrease in the normal-Ca group. There was no significant difference observed in time-to-peak tension value between the two groups. As distinct from time-to-peak tension, the time-to-peak fluorescence was significantly less at any non-zero preload than at L_0,

regardless the $[Ca^{2+}]_o$ (Figure 4B). No difference in the time-to-peak fluorescence was obtained between the two groups as well as between different preload ranges within the same group.

The result indicates that opposite preload-dependent changes of time-to-peak tension and time-to-peak fluorescence exist in rat ventricular myocardium: time-to-peak tension increased with preload whereas time-to-peak fluorescence decreased.

3.4 Effect of preload on tension relaxation time and fluorescence decay

The mechanical relaxation progressively and monotonically decelerated with preload in both hypo-Ca and normal-Ca groups, regardless of the time estimation level (Figure 5A and 5B). In hypo-Ca each 10% L_0 muscle stretch resulted in growth of T_{20}, T_{50} and T_{80} with gains of 1.24±0.01, 1.26±0.01 and 1.27±0.02 times respectively (means were not significantly different). In normal-Ca group the muscle stretch caused the increase of T_{20}, T_{50} and T_{80} with gains of 1.27±0.02, 1.26±0.03 and 1.24±0.05 times respectively. No significant difference in this increase for any particular preload range was found between the two groups.

The preload-induced increase of relaxation time in hypo-Ca group was higher for the early phase of relaxation (Figure 5A). Thus, for muscle stretch to ~120% L_0 the increase was higher for T_{80} (163±4% of value at L_0) and smaller for T_{20} (153±6%). In normal-Ca group this preload-induced increase of relaxation time was higher for early relaxation phase only at lengths up to 115% L_0 while for large preloads this increase was higher for late phase of relaxation (Figure 5B). For muscle stretch to ~120% L_0 the observed increase was 167±5% for T_{20} and 159±3% for T_{80}.

The time needed to fall from peak fluorescence to estimated level of the fluorescence amplitude was preload-dependent with more complex behavior (Figure 5C and 5D). For the early phase of fluorescence decay this time decreased with stretch, while progressively increased for the late phase of this decay (compare light and dark grey symbols in Figure 5C and 5D). For levels near T_{50} the decay time showed negligible preload-dependence. Such effect of preload on the fluorescence decline time was obtained in all investigated muscles and showed no principal difference between hypo-Ca and normal-Ca groups.

3.5 Distinct effect of preload on twitch and calcium transient

The superposition of active tension or fluorescence traces, obtained in steady-state conditions at different preloads, is shown on Figure 6. The traces are normalized to their peak values to represent the preload-dependent changes in relaxation phase more clearly. Tension relaxation progressively decelerated with increase of preload, resulting in marked prolongation of total twitch duration regardless of early or late phase of relaxation is examined (Figure 6A, black arrows). In contrast, a preload-dependent change in the rate of fluorescence decline depends on which phase of Ca^{2+} transient is considered. An increase of preload resulted in a progressive rise of the rate of fluorescence decline in the initial phase and progressive slowing down of that rate in the late phase (as indicated by black arrows in Figure 6B). Superposition of fluorescence traces, obtained at different preloads and normalized to their respective amplitudes, revealed their intersection at the point approximately on half of the trace (Figure 6B,

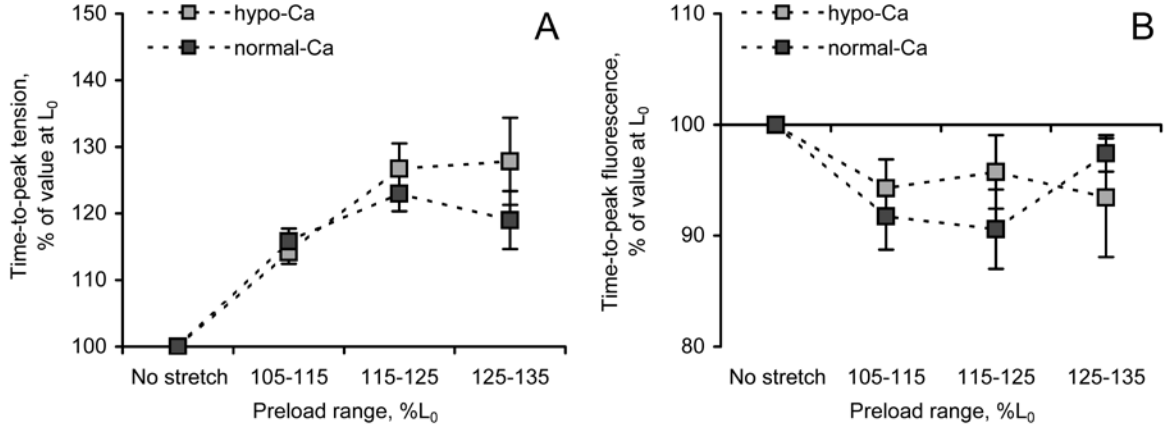

Figure 4. Preload-dependent changes in time-to-peak tension and fura-2 fluorescence. Preload-dependent changes (expressed as % of value at L_0) in time-to-peak tension (A) and time-to-peak fura-2 fluorescence (B) are shown. Data are obtained for hypo-Ca group (light grey bars) and normal-Ca group (dark grey bars). In both groups a value at any non-zero preload significantly differs from the corresponding value at L_0.

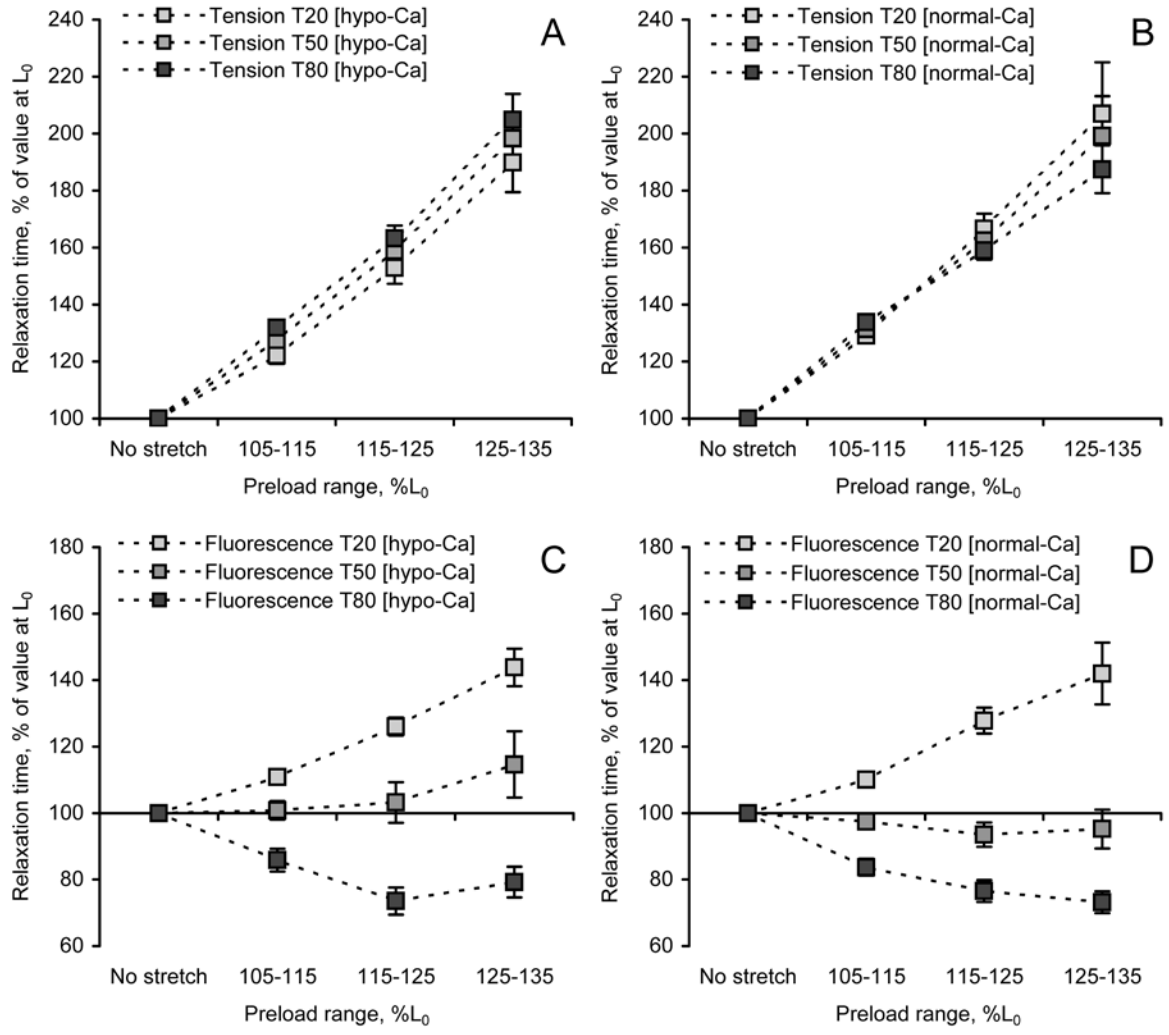

Figure 5. Preload-dependent changes in mechanical relaxation and fura-2 fluorescence decay time. Preload-dependent changes (expressed as % of value at L_0) in active tension relaxation time from peak value to 20% (Tension T20), 50% (Tension T50) and 80% (Tension T80) of the peak (A and B) and fura-2 fluorescence decay time from peak value to 20% (Fluorescence T20), 50% (Fluorescence T50) and 80% (Fluorescence T80) of the fluorescence amplitude (C and D) are shown. Data are obtained for hypo-Ca group (panels A and C) and normal-Ca group (panels B and D). See text for details.

white arrow). This effect was observed in each muscle studied in this work, regardless to $[Ca^{2+}]_o$ in washing solution.

Further, an increase of preload causes a brief delay in Ca^{2+} transient decline (named as "bump" in [28]); the higher preload the more prominent "bump" (Figure 7A, from top to bottom, indicated by arrows). As it is shown in Figure 7A, there is monotonic decline of fluorescence from peak to diastolic level, which was virtually independent on preload (thick black lines). At the same time, muscle stretch caused appearance of the "bump" on decay phase of fluorescence trace, which became more prominent with an increase in preload and disappeared at slack length (Figure 7A, compare thin lines on bottom and top panels). Preload changes not only the amplitude (intensity) of the "bump" but also changes its timing properties, e.g. time of peak deviation of fluorescence trace from monotonic decline increased with preload (Figure 7B).

We assessed the "bump" area (expressed as a % of the total area under the corresponding fluorescence trace, minus area of the "bumped" part) in both hypo-Ca and normal-Ca groups. Muscle stretch led to the significant increase of this relative area in both groups. This increase was significantly higher (with a gain twice as large) in the hypo-Ca group compared with normal-Ca group, at any studied preload range (Figure 7C, $P<0.05$).

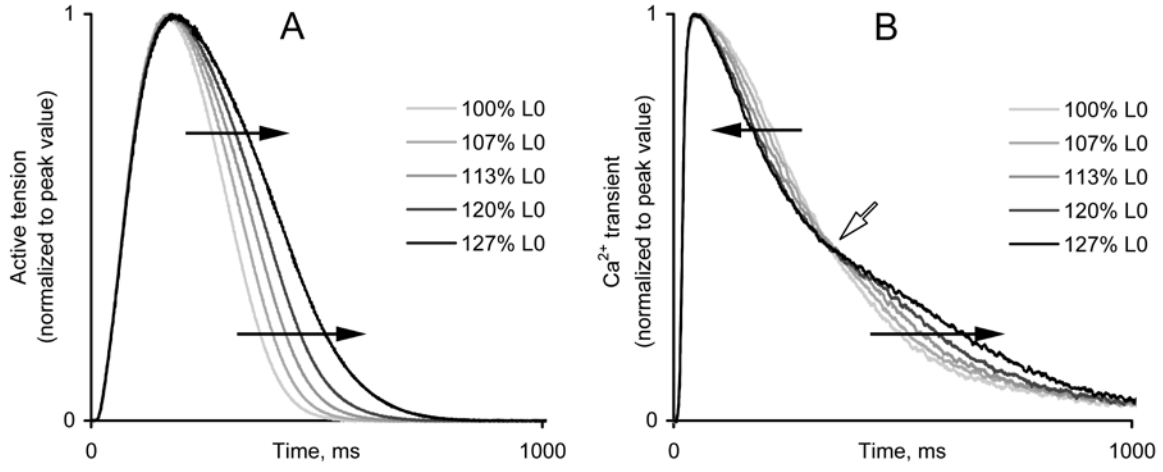

Figure 6. "Dissociation" between preload-induced changes in active tension and Ca^{2+} transient. The superposition of active tension traces (A) and fura-2 fluorescence traces (B) normalized to their respective peak values is shown. Data are obtained in rat RV papillary muscle at different preloads. Black arrows indicate the direction of trace displacement with increase of muscle length (preload). Note that increase of preload causes a slowing down in the mechanical relaxation at any phase (initial or late, panel A), whereas fluorescence decay accelerates in the initial phase and decelerates in the late phase with the corresponding preload change (panel B), which leads to the "crossover" of the fluorescence traces obtained at different preloads (as indicated with white arrow).

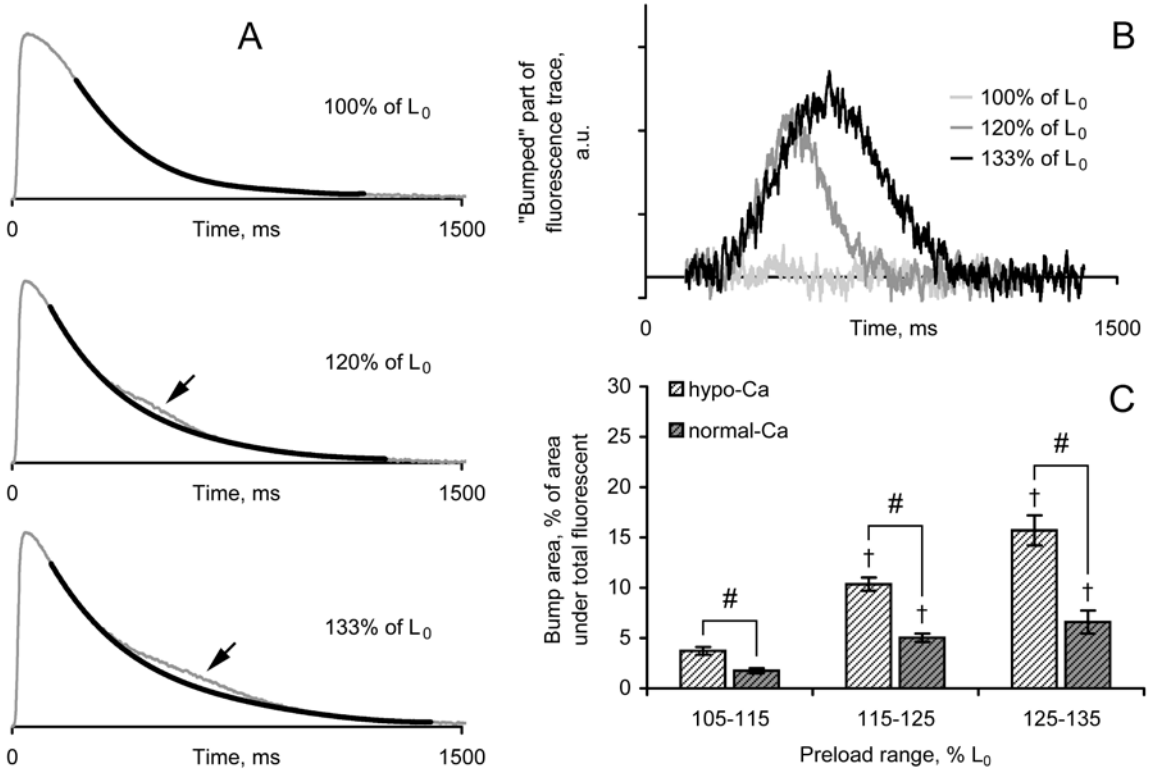

Figure 7. Preload-induced change in "bump" on fura-2 fluorescence decay phase. Panels A: typical fluorescence traces recorded in a muscle tested at increased preloads (from top to bottom). Note that the "bump", appeared on fluorescence decay phase and indicated with black arrow, increased with preload. Thin grey line – original fluorescence trace, thick black line – monotonic part of the fluorescence decline. Panel B: superposition of the "bump" traces calculated as the difference between raw fluorescence trace and monotonic decline at corresponding preload (the same data as on the panels A). C: preload-dependent changes in the area of the "bump" expressed in a % of the area under the corresponding fluorescence trace assuming to be declined monotonically. Data are obtained for hypo-Ca group (light grey bars) and normal-Ca group (dark grey bars). # - significant difference between hypo-Ca and normal-Ca groups for the given preload range; † - significant difference from preload range of 105-115% of L_0 within the same group; $P<0.05$.

4. Discussion

The main finding of this study is that preload-dependent changes in mechanics are mainly "dissociated" from the concomitant changes in free cytosolic calcium in the same preparation of rat right ventricular papillary muscle. Similar findings were made in numerous studies on warm-blooded animal and human myocardium [9,16-24]. The preload-induced interplay between cytosolic calcium and force development is thought to be indirect and most likely involves cooperative effects of association/dissociation of Ca-TnC [2,4,6,7,30,31].

4.1 Effect of preload on peak active tension and fluorescence

We did not observe, in general, a mutual effect of preload on the peak isometric active tension and amplitude of Ca^{2+} transient and found that the effect depends on $[Ca^{2+}]_o$ (as shown in the behavior of the grey symbols in Figure 1A and 1B). The "dissociation" between preload-dependent changes in mechanics and calcium handling has been observed in numerous studies carried out on mammalian and human myocardium [11,12,21,24,32-35]. It has been shown in cat [18], rat [16,17,19], mouse [9] and rabbit [20,21] that muscle stretch is not accompanied by increase of Ca^{2+} transient amplitude immediately after the stretch but does produce a slow increase. Peak active force, however, increased immediately after stretch and then rose additionally during several minutes.

Our findings indicate that the preload-dependent *relative* increase in Ca^{2+} transient amplitude is markedly higher at low than at physiological $[Ca^{2+}]_o$. The contribution of different preload-dependent cellular and/or intracellular sources of additional Ca^{2+}, e.g., stretch-activated channels (SAC) [9,33], L-type calcium channels [36,37] or ATP activity of myosin cross-bridges [38] might depend on the level of $[Ca^{2+}]_o$. At low $[Ca^{2+}]_o$ the *relative* contribution of these sources to the total amount of free cytosolic Ca^{2+} would be higher resulting in *relatively* larger preload-dependent rise of Ca^{2+} transient amplitude, compared with physiological $[Ca^{2+}]_o$. Possible contribution of Ca-TnC kinetics into the observed changes seems to be negligible because Ca^{2+} transient reaches its peak when developed force is quite far from peak value.

4.2 Effect of preload on diastolic level of tension and fluorescence

It is well known that stretch causes the increase of passive tension in health myocardium, while the changes in resting $[Ca^{2+}]_i$ level reported by different investigators are dissimilar. No preload-dependence of resting $[Ca^{2+}]_i$ has been observed in stimulated ferret and mouse cardiac muscle [8,9] and rat atrial or ventricular myocyte [13,26]. On the other hand, stretch of rat trabeculae resulted in a decrease in resting Ca^{2+} level [9,17], while stretch of stimulated Guinea-pig trabeculae or isolated myocyte produced a rise in resting $[Ca^{2+}]_i$ level [10-12,15]. In the present study we observed the preload-induced rise of the resting Ca^{2+} in both low and physiological levels of $[Ca^{2+}]_o$.

One of the possible explanations of the observed change in resting $[Ca^{2+}]_i$ is that stretch promotes opening of nonspecific cationic SAC, rise of Na^+ entering to a cell, and rise of cytosolic Ca^{2+} due to increased function of NCX in reverse mode. It has been shown that application of streptomycin, which blocks SAC on cell membrane, or inhibition of Na^+-H^+ exchanger abolishes preload-dependent changes in resting $[Ca^{2+}]_i$ in mouse [9,14,39] and Guinea-pig myocardium [33]. In contrast, inhibition of Ca-release from sarcoplasmic reticulum (SR) by ryanodine leads to more apparent preload-dependent changes in stimulated rat ventricular trabeculae [16].

4.3 Effect of preload on time-to-peak tension and fluorescence

There is evidence that the time-to-peak of Ca^{2+} transient is unchanged or even decreased with preload in mammalian myocardium [16,18,22,23], whereas the time-to-peak of active tension significantly increased, as we observed is consistent with [18,22] and others. Our data indicate that both in hypo-Ca and normal-Ca solution the Ca^{2+} transient time-to-peak was significantly smaller at any stretch than at slack length L_0, while no further preload-dependent change in the parameter was observed. One of the possible reasons is that our experimental system has 2.5 msec time resolution of the fluorescence sampling, which is as large as 5% of the value of Ca^{2+} transient time-to-peak. The preload-dependent changes in time-to-peak of fluorescence would be insufficiently large to be detected in our system. Another possible explanation might involve rapid acceleration of Ca-TnC formation in cardiac muscle at even light stretch, which causes preload-dependent increase of calcium binding to TnC and corresponding decrease of Ca^{2+} transient time-to-peak. The permanence of Ca^{2+} transient time-to-peak observed at different preloads might reflect nonlinear dependence of the parameter on preload.

4.4 Effect of preload on relaxation time of tension and fluorescence

We found that the relaxation of force and decline phase of Ca^{2+} transient are mostly subjected to preload, in

a distinct way (as shown in Figure 6A and 6B). The preload-dependent changes of the rate of Ca^{2+} transient decline were dependent on the phase of this decline. In particular, the increase of preload caused consistent acceleration of Ca^{2+} transient decline in the initial phase but slowing down in the late phase. At the same conditions, the mechanical relaxation increased with preload regardless of the phase of relaxation.

Our results are partially consistent with data from others. It has been shown that the duration of Ca^{2+} transient gradually decreased with preload, whereas the duration of mechanical response increased [12,16,18,22]. The superposition of Ca^{2+} transients obtained for rat trabeculae at low and high preload showed that the traces have "cross-over" approximately on half of the amplitude; at high preload the Ca^{2+} transient decays faster in the initial phase and slower in the late phase [16,17,23]. Application of BDM significantly decreased preload-dependent change of Ca^{2+} transient decay rate in ferret [22] and rat [27] whereas increase of $[Ca^{2+}]_o$ caused rise of the decay in a way similar to increase of preload [22,28]. These results indicate that the changes are mediated by Ca-TnC association/dissociation kinetics rather than by other mechanisms, e.g. stretch-activated channels [27]. On the other hand, no preload-dependent changes of Ca^{2+} transient decay in rabbit [21] and Guinea-pig [29] has been reported.

Our results may be explained by the assumption that Ca-TnC kinetics participates in the observed preload-dependent changes in Ca^{2+} transient decline. Ca^{2+} binds to TnC more rapidly and tightly in stretched muscle due to the co-operative attachment of myosin cross-bridges and higher affinity of TnC to Ca^{2+} [12]. This causes faster decline of Ca^{2+} transient in its initial phase, compared with low preload [12,16]. On the other hand, the rate of Ca-TnC dissociation at high preload is decreased (again, due to augmented affinity of TnC to calcium) and this evokes prolongation of Ca^{2+} transient decline in its late phase [16].

Moreover, the comparison of data presented on Figure 5C and 5D shows that at low $[Ca^{2+}]_o$ the decline time which demonstrates minimal preload-dependence is lower than at physiological $[Ca^{2+}]_o$. It is well known that the rate of formation of Ca-TnC complexes as well as the lifetime of a complex is strongly regulated by the cooperative effect [4]. Lowering of $[Ca^{2+}]_o$ would lead to a decrease in free cytosolic calcium and, in turn, the cooperativity and total amount of Ca-TnC complexes. As a result, TnC would release Ca^{2+} more easily due to a reduced affinity and Ca^{2+} transient decay in its initial phase in stretched muscle accelerates relative to the Ca^{2+} transient of un-stretched muscle to a lesser degree than at physiological $[Ca^{2+}]_o$.

4.5 "Bump" on the Ca^{2+} transient

We observed in our study that an increase of preload caused brief delay in Ca^{2+} transient decline and "bump"-like configuration on the fluorescence trace in rat papillary muscle. At slack length the "bump" was not observed in both hypo-Ca and normal-Ca groups of muscles. Moreover, in our test measurements we found that application of 5 mM BDM completely abolishes the "bump" and leads to the lack of preload-dependence of both initial and late phases of Ca^{2+} transient. These findings indicate the direct contribution of Ca-TnC kinetics into the observed preload-dependent changes in Ca^{2+} transient decline.

Similar findings about the contribution of Ca-TnC kinetics to the "bump" have been reported by others [16,28]. Thus, gradual increase of BDM concentration in washing solution caused progressive vanishing of "bump" in isometrically contracting rat trabeculae with no detectable changes in other phases of Ca^{2+} transient; the "bump" disappeared completely at 10 mM BDM [28]. Sudden release (which is thought to change Ca-TnC kinetics rapidly) of a cardiac muscle bathed in BDM-free solution produced a brief slowing down of Ca^{2+} transient decay together with formation of "bump", due to extensive release of Ca^{2+} from TnC, whereas BDM application abolished the "bump", as it has been shown in cat [18], rat [23,28] and ferret papillary muscle [40]. The shape and quantitative properties of the "bump" evoked by the sudden release were similar to that observed in isometric twitch under high preload thus suggesting the same mechanism is involved in these two conditions [18].

It has been found that both the amplitude of Ca^{2+} transient and intensity (amplitude) of the "bump" increased in a dose-dependent manner with $[Ca^{2+}]_o$ in rat right ventricular trabeculae [28]. The authors reported that "bump" amplitude correlates with average rate of Ca^{2+} transient decline: the higher rate (the shorter Ca^{2+} transient) the larger "bump" is observed. We found that the preload-induced *relative* increase in the "bump" area (not amplitude) correlates inversely with $[Ca^{2+}]_o$ concentration: at low Ca^{2+} in saline solution the increase is higher compared with physiological $[Ca^{2+}]_o$. This result might indicate that the total amount of Ca^{2+} released from TnC during Ca^{2+} transient decline depends primarily on the rate of Ca-TnC association/dissociation but not on the Ca^{2+} level in cytosol.

5. Conclusions

The characteristics of single twitch and Ca^{2+} transient in isolated rat right ventricular papillary muscle are preload-dependent. However, these changes in force

development/relaxation and free cytosolic calcium kinetics are "dissociated" from each other. The plausible intermediate link between the mechanical response and free calcium handling in cardiac cell is the kinetics of association/dissociation between Ca^{2+} and troponin C complex. This kinetics is thought to be an important contributor to the preload-induced changes in the rate of Ca^{2+} transient decline and thus may be the main mechanism of the generation of the "bump"-like phase on Ca^{2+} transient in rat cardiac muscle.

A level of extracellular calcium modifies preload-dependent *relative* changes in Ca^{2+} transient characteristics, like amplitude or "bump", but not in active tension development and relaxation. It might be concluded that the level of $[Ca^{2+}]_o$ *per se* is not particularly important for the observed *relative* changes in the mechanical response in rat myocardium during the action of the Frank-Starling Law.

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