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Aleksandra Paterek, Marta Kępska, Joanna Kołodziejczyk, Michał Mączewski, *Urszula Mackiewicz

The effect of pacing frequency on the amplitude and time-course of cardiomyocyte shortening in isolated rat cardiomyocytes

Wpływ częstości drażnienia na amplitudę i kinetykę skurczu w izolowanych kardiomiocytach szczura

Department of Clinical Physiology, Centre of Postgraduate Medical Education, Warsaw
Head of Department: Michał Mączewski, MD, PhD

Keywords

isolated cardiomyocytes, frequency of pacing, myocyte shortening, time to peak of contraction, time to relaxation

Sowa kluczowe

izolowane kardiomiocyty, częstotliwość drażnienia, amplituda skurczu, czas skurczu, czas rozkurczu

S u m m a r y

Introduction. Isolated adult cardiomyocytes are an important research tool in the field of pathophysiology of the cardiac muscle. Isolated myocytes are electrically paced to elicit their contractile function. In the majority of cases, the pacing frequency used in *in vitro* experiments is 1 Hz, while physiological heart rate in rodent heart (the most common source of isolated cardiomyocyte) is about six times higher (approx. 6 Hz).

Aim. To compare the amplitude and kinetics of contractile signal in myocytes paced at 1 Hz (the most often used frequency of pacing *in vitro*) with these parameters measured in cardiomyocytes paced at frequency closer to physiological one for a rat heart (4-8 Hz).

Material and methods. Cardiomyocytes were isolated by enzymatic digestion from hearts of 3 months old male Wistar-Kyoto rats. The myocytes were electrically paced at 1 Hz, subsequently the frequency of pacing was changed to 4 Hz and gradually increased up to 8 Hz, every 10 sec. Sarcomere shortening was registered as a measure of myocyte contractile performance, by a video-edge detection system. The amplitude and time-course of the signal were analysed.

Results. We did not find the difference between the amplitude of contraction at 1 Hz and at higher frequencies (4-8 Hz). Both the time of contraction development (time-to-peak) and relaxation time decreased with increasing pacing frequency. The contribution of the contraction and relaxation phases to the total duration of the cycle did not change after increasing the frequency of pacing to 4 Hz. However, relative lengthening of the contraction phase was observed for higher frequencies, but it did not exceed a few percent.

Conclusions. These results indicate that in adult cardiomyocytes isolated from a heart of a young healthy rat, the parameters of contraction-relaxation cycle at low pacing frequency (1 Hz) used in *in vitro* experiments surprisingly closely correspond with those measured at the frequency near the physiological heart rate.

S t r e s z c z e n i e

Wstęp. Izolowane z serca szczura kardiomiocyty są ważnym narzędziem w badaniach nad patofizjologią mięśnia sercowego. W celu indukcji czynności skurczowo-rozkurczowej in vitro kardiomiocyty są stymulowane impulsami elektrycznym. Najczęściej używana w tych badaniach częstotliwość stymulacji wynosi 1 Hz, podczas gdy fizjologiczna częstotliwość pobudzania kardiomiocytów w sercu szczura jest około 6-krotnie wyższa.

Cel pracy. Porównanie parametrów opisujących amplitudę i kinetykę skurczu w izolowanych kardiomiocytach pobudzanych z częstotliwością 1 Hz z parametrami zmierzonymi w kardiomiocytach pobudzanych z częstotliwością leżącą w zakresie częstotliwości fizjologicznych dla serca szczura (4-8 Hz).

Materiał i Metody. Kardiomiocyty zostały wyizolowane z serc 3-miesięcznych szczurów płci męskiej, rasy Wistar-Kyoto metodą enzymatyczną. Pozyskane komórki były stymulowane impulsami elektrycznymi z częstotliwością 1 Hz. Po uzyskaniu stabilnych rejestracji częstotliwość drażnienia była zwiększana do 4 Hz i następnie sukcesywnie zwiększana co 10 s do 8 Hz. Rejestrowano skrócenie sarkomeru jako miarę aktywności skurczowej kardiomiocytów za pomocą systemu detekcji krawędzi sarkomeru. Analizowano amplitudę i przebieg czasowy sygnału.

Conflict of interest
Konflikt interesów

None
Brak konfliktu interesów

Address /adres:

*Urszula Mackiewicz
Department of Clinical Physiology
Centre of Postgraduate Medical Education
Marymoncka 99/103, 01-813 Warszawa
tel.: +48 (22) 569-38-41,
fax: +48 (22) 569-37-12
urszula.mackiewicz@cmkp.edu.pl

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Wyniki. Amplituda sygnału nie różniła się istotnie statystycznie w badanym zakresie częstotliwości. Czas narastania skurczu i opadania skurczu uległy istotnemu skróceniu wraz ze wzrostem częstotliwości drażnienia. Jednak względny udział fazy skurczu i rozkurczu w całkowitym czasie trwania cyklu skurczowo-rozkurczowego nie zmienił się przy wzroście częstotliwości z 1 Hz do 4 Hz. Niewielkie, względne wydłużenie fazy skurczu (o kilka procent) zostało zaobserwowane dla częstotliwości wyższych niż 4 Hz.

Wnioski. Otrzymane wyniki wskazują, że w kardiomiocytach izolowanych z serc zdrowych młodych szczurów, parametry skurczu rejestrowanego przy częstotliwości 1 Hz, najczęściej używanej w badaniach *in vitro*, zaskakująco dobrze odzwierciedlają te zmierzone przy częstotliwościach fizjologicznych dla serca szczura.

INTRODUCTION

Isolated adult cardiomyocytes are an important research tool in the field of pathophysiology of the cardiac muscle. The first techniques of cardiomyocyte isolation were introduced over 30 years ago and up till now numerous unique data, hardly available in an intact heart muscle, concerning cellular and sub-cellular physiology and pathology were provided, especially in the area of cellular electrophysiology, intracellular Ca^{2+} handling, and cardiomyocyte mechanics. Moreover, a significant piece of information about protein expression, function and localisation was obtained through the application of molecular biology techniques in isolated cardiomyocytes. Finally, elaboration of the methods of short-term myocyte culture gave way to a wide range of toxicological studies as well as provided the possibility of genetic manipulations in intact cardiomyocyte through viral-based gene delivery or gene silencing (1).

In spite of indisputable advantages of using the isolated adult cardiomyocytes, several limitations of this model should be underlined. Firstly, isolated cardiomyocytes are deprived of their natural environment securing tight coupling with other myocytes and components of extracellular matrix. This certainly affects the mechanical tension (strain) rising in myocytes during contraction-relaxation cycle. The contraction of the cardiomyocyte *in vivo* is activated against forces generated in extracellular matrix and other surrounding cardiomyocytes. In contrast, the isolated cardiomyocytes are unloaded. To some extent this difference may, at least partly, be compensated because isolated cardiomyocytes tend to adhere to the glass bottom of superfusion chamber and thus contract against adhesive strength. However, significant differences of myocyte loading exist between cells contracting *in vitro* and *in vivo* that may influence their contractile properties (2).

Secondly, the cardiomyocytes after isolation are perfused with solutions that should imitate physiological condition in respect to ions and other substrates concentration as well as to temperature. Composition of the perfusion solution applied during experiments vary depending on the laboratories. However, generally the energy comes from glucose added to the superfusion solution, while in cardiomyocyte *in vivo* from fatty acids. It may modulate cardiomyocyte metabolism and thus ATP synthesis, the energy source for contractile activity (2).

The temperature of perfusion solutions in most of the current studies is kept at the physiological level, however – in earlier papers – cardiomyocytes were perfused at room temperature, in which case the amplitude was much higher and kinetics slower than at the physiological one (3).

Finally, the frequency of pacing is a very important factor (4). To force contraction in isolated myocytes, electrical pulses are provided to the myocyte bathing solution by the electrodes immersed in the solution. The frequency of the applied pulses is rather low and depends on the paper, it starts from very low frequencies (0.2-0.5 Hz) to medium ones (3-4 Hz). Most often, the frequency used in *in vitro* experiments is 1 Hz, while physiological heart rate in rodent heart (the most common source of isolated cardiomyocyte) is about six (rat) or nine (mouse) times higher (about 6 and 9 Hz, respectively) (5-7).

The low frequency of *in vitro* pacing ensures more stable recordings without spontaneous contractions often observed after application protocols with fast pacing. Moreover, recordings at low frequency are preferred due to clear baseline between individual contractions, providing a more reliable analysis of contractile signal, especially its time-course (fig. 1).

It is well known that increase in the pacing frequency results in reduction of contraction and relaxation time, however, to the best of our knowledge, this phenomenon has not been systematically studied in rat isolated myocytes especially in a wide range of frequencies. In particular, no results are available for higher frequencies (5-8 Hz) typical of a rodent heart (6, 7). Investigations of the impact of the pacing rate on the amplitude of contraction (force-frequency relationship) in isolated cardiomyocyte are also rare and the results are inconsistent (4, 5).

Thus, the aim of our study was to investigate the effect of pacing frequency on the amplitude, time of relaxation and contraction and their contribution to duration of the whole contraction-relaxation cycle in adult cardiomyocytes isolated from the left ventricle of a rat heart. We aimed to compare these parameters in myocytes paced at 1 Hz (the most often used frequency of pacing in vitro) with these parameters measured in cardiomyocytes paced at the frequency closer to physiological (4-8 Hz). This comparison will make it pos-

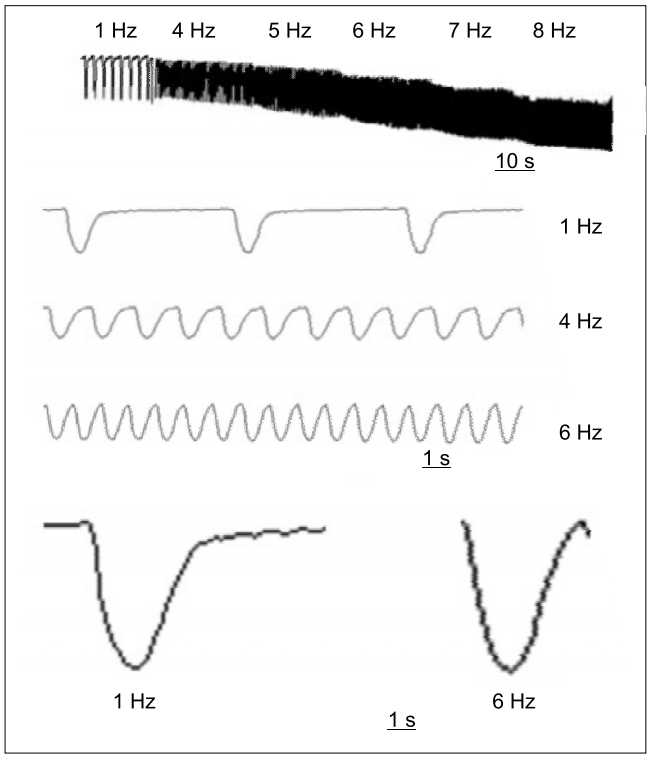

Fig. 1. Representative recording of sarcomere shortening in myocyte paced at 1 Hz and at 4-8 Hz for different time scale

sible for us to conclude whether the result obtained for lower frequencies may, at least in respect to amplitude and time-course of contraction, mirror the contractile parameters in an intact heart. The results may also give indication concerning the pacing protocols in isolated cardiomyocytes to better mimic *in vivo* conditions.

MATERIAL AND METHODS

Experiments were performed in enzymatically isolated ventricular myocytes of the rat heart. Five 3 months old male Wistar-Kyoto rats, weighing 270-300 g, were used in the study. The study conformed to the Guide for the Care and Use of Laboratory Animals, US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local ethics committee.

Myocyte isolation

Rats were anaesthetised with pentobarbital sodium (50 mg/kg body weight, ip) and their hearts were excised rapidly. The aorta was cannulated and retrogradely perfused for 5 minutes with nominally Ca^{2+}-free solution containing 100 μmol/l of EGTA of the following composition (mmol/l): 144 NaCl, 5 KCl, 1 MgCl2, 0.43 NaH2PO4,10 N-2-hydroxyethylpiperazine-N˘-2-etanesulfonic acid (HEPES), 11 glucose and 5 sodium pyruvate. The pH of the solution was adjusted with NaOH to 7.3. Initial washout period was followed by 10-20 minutes of perfusion with Ca^{2+}-free Tyrode solution containing 15 mg collagenase B (Roche Diagnostics) and 3 mg protease (Sigma) per 50 ml. Thereafter, the left ventricle was separated and placed in vessels with enzymes containing Tyrode's solution and disrupted into small strands. The cell suspension was filtered through a nylon mesh, and left to sediment. The supernatant was discarded and cells were washed twice with Tyrode's solution, the Ca^{2+} concentration being increased gradually to 1 mmol/l (8).

Myocytes were placed in the superfusion chamber mounted on the stage of an inverted microscope (Zeiss), perfused with Tyrode's solution containing 1.8 mmol/l Ca^{2+} at 37°C.

Myocyte contractions recording

The myocytes contractions were recorded by a video-edge detection system including a fast digital dimensioning video camera (IonOptix LLC, Milton USA) enabling acquisition of the cell and sarcomere length changes in real-time.

Myocytes contractions were elicited by electrical pacing at 1 Hz. After achieving the contraction steady-state, the frequency of pacing was changed into 4 Hz and gradually increased up to 8 Hz, every 10 sec. (fig. 1).

Sarcomere shortening was taken as a measure of myocyte contractile performance due to more stable registrations at sarcomere then at myocyte level, especially at higher pacing frequencies. Amplitude and time-course of the signal were analysed by the IonWizard software (IonOptix). Contraction amplitude was expressed as the difference between systolic and diastolic sarcomere length and normalised as a percentage of the resting sarcomere length. The contraction time (time-to-peak) was calculated as the time from the initiation of contraction to the maximal sarcomere shortening. The time required for re-lengthening sarcomere to 90% of resting sarcomere length was taken as the relaxation time.

Statistical analysis

Statistical analyses were performed with SigmaPlot v.11.0 software. Differences between means were evaluated by one-way ANOVA followed by Tukey's post-hoc test or Kruskal-Wallis test. In the case of two groups, comparison with the Student's t-test was used.

The comparison of the means was made between 1 Hz and higher frequencies (4-8 Hz) as well as between 6 Hz and other frequencies (1 Hz, 4-5 Hz, 7-8 Hz). Data were presented as means ± SE. Differences were considered significant if $p < 0.05$.

RESULTS

The amplitude of contraction

The amplitude of myocyte shortening measured at sarcomere level in myocytes paced at 1 Hz was about 8% and did not change significantly along with the increase in the pacing frequency. Some decrease in the mean sarcomere shortening was observed at frequencies higher than 6 Hz, however it was statistically insignificant (fig. 2a).

Time-to-peakcontraction

The time-to-peak contraction was about 100 ms and progressively decreased with increasing pacing

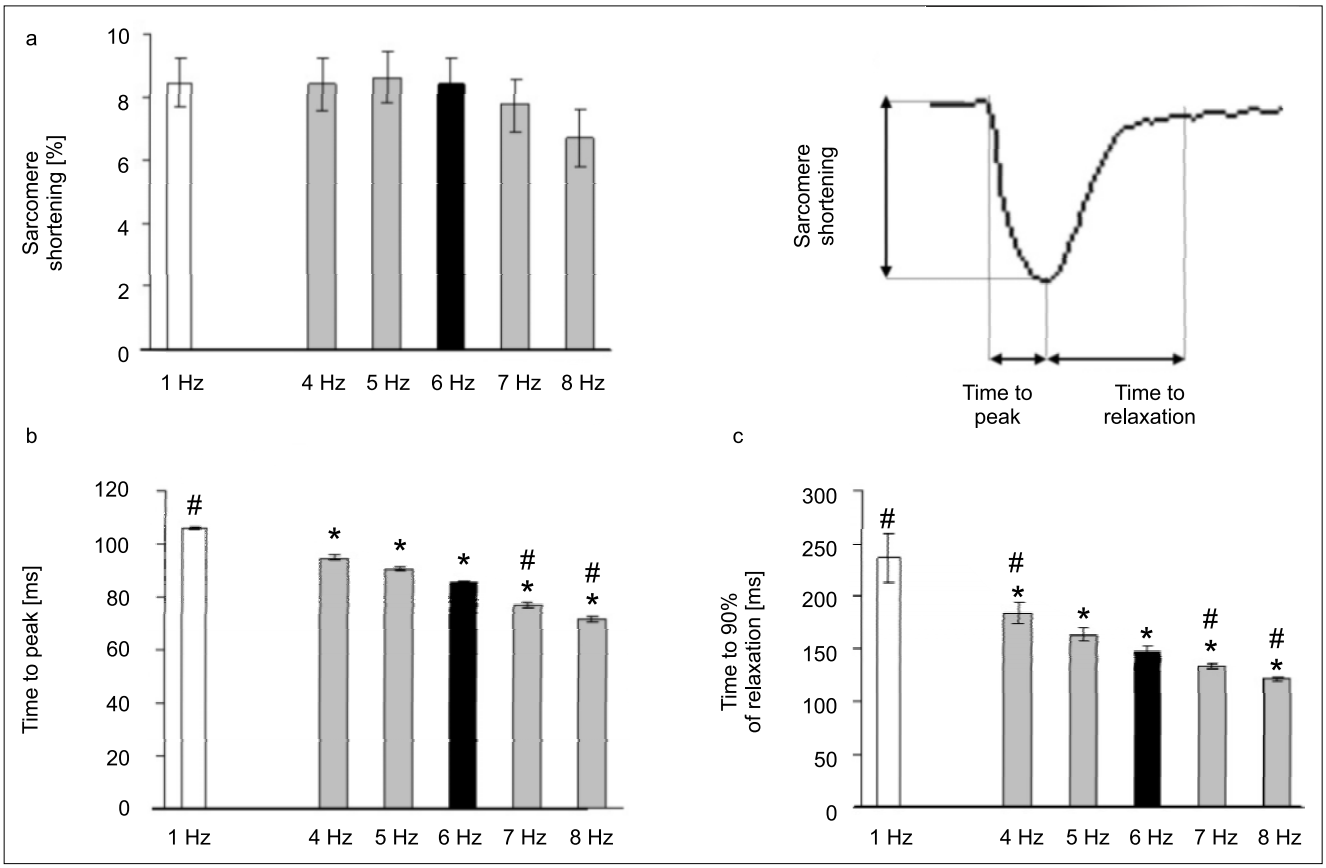

Fig. 2. The parameters of sarcomere shortening in isolated ventricular myocytes electrically paced at 1 Hz and at 4-8 Hz, a – amplitude of sarcomere shortening, b – time-to-peak of contraction and c – time to 90% of relaxation. Means ± SEM from n = 25 myocytes from 5 hearts in each group. *p < 0.05 comparisons were made vs. 1 Hz; #p < 0.05 comparisons were made vs. 6 Hz

frequency and at all pacing frequencies (4-8 Hz) was significantly shorter then for 1 Hz. At 6 Hz, time-to-peak was shorter by about 32 % than in myocytes paced at 1 Hz. There were no differences between time-to-peak at 6 Hz and 4 as well as 5 Hz, however for 7 and 8 Hz the time-to-peak was shorter than at 6 Hz (fig. 2b).

Relaxation time

The relaxation time was about 225 ms in myocytes paced at 1 Hz. The decrease in the relaxation time was pronounced at higher frequency of pacing and at 6 Hz relaxation time was about 70% shorter than in myocytes paced at 1 Hz. As compared to 6 Hz relaxation time was significantly longer for 1 and 4 Hz and shorter for 7 and 8 Hz. There were no statistically significant differences with regard to this parameter for 6 and 5 Hz.

The relative contribution of the duration of contraction and relaxation phases to the contraction-relaxation cycle

The time of contraction development (time-to-peak) accounted for about 33% of the total duration of contraction-relaxation cycle, while the relaxation occupied about 67% of the cycle in myocytes paced at 1 Hz. The contribution of the contraction and relaxation phases to the total duration of the cycle did not change after increasing the frequency of pacing to 4 Hz. However, relative lengthening of the contraction phase was observed for higher frequencies. At 6 Hz, relative contribution of contraction phase to the contraction-relaxation cycle increased to 36%, while contribution of relaxation decreased to 64% (fig. 3).

DISCUSSION

In this paper, we investigated the effect of pacing frequency on the amplitude and time-course of contractions registered in isolated rat ventricular cardiomyocytes to check if these parameters measured at low frequency, the most often used in the *in vitro* experiments, correspond with these parameters measured at frequencies close to the physiological range for rats. We found that the amplitude of sarcomere shortening, a measure of contractile force, did not differ for a wide range of pacing frequencies (1-8 Hz). The duration of contraction and relaxation phases was frequency-dependent and progressively decreased with increasing the pacing frequency. Shortening the relaxation phase was more pronounced than the contraction one, resulting in a slight increase (by about 3%) in relative contribution of contraction phase to the duration of the whole contraction-relaxation cycle at frequencies higher than 5 Hz. These results show that in adult cardiomyocytes isolated from a healthy heart of a young rat the parameters of contraction-relaxation cycle at the low pacing frequency (1 Hz) surprisingly closely correspond with those measured at the frequency close to physiological heart rate (6 Hz).

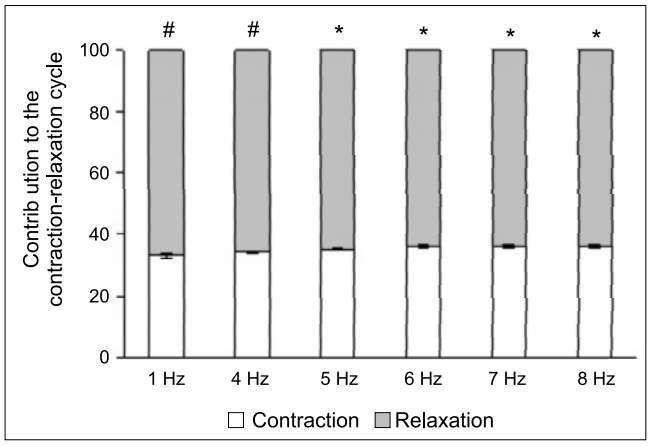

Fig. 3. Relative contribution of contraction and relaxation in the contraction-relaxation cycle in isolated ventricular myocytes electrically paced at 1 Hz and at 4-8 Hz Means ± SEM from n = 25 myocytes from 5 hearts in each group. *p < 0.05 comparisons were made vs. 1 Hz; #p < 0.05 comparisons were made vs. 6 Hz

In the heart muscle, apart from the Frank-Starling mechanism, the strength of the contraction is regulated by heart rate. This regulation provides an intrinsic mechanism essential for the immediate adjustment of cardiac contractile function to rapid changes of body requirements. In response to the increase in the heart rhythm, accumulation of Na^+ ions in cardiomyocytes takes place due to insufficient activity of Na^+/K^+ ATP-ase. Subsequently to intracellular accumulation of Na^+ outward Ca^{2+} transport through Na^+/Ca^{2+} exchanger is diminished, which results in the increase in intracellular concentration of Ca^{2+} ions, activation of sarcoplasmic reticulum Ca^{2+} ATP-ase (SERCA), increase in Ca^{2+} transport to the sarcoplasmic reticulum (SR) and SR Ca^{2+} content. Finally, more Ca^{2+} is released from the SR and contractile force increases. This is accompanied by faster relaxation and ensures appropriate ventricular filling at high heart rates (9).

Positive force-frequency relation takes place in a mammal heart, including humans. It has been shown in muscle preparation from a human healthy heart, in the physiological range of pacing frequencies (1-2.5 Hz). In contrast, in a failing heart, flat or even negative force-frequency relation was observed mainly due to abnormal Ca^{2+} handling, especially diminution of SERCA activity and expression. In this context, interventions reducing heart rate in a failing heart may be beneficial due to increase in the contractile force of individual cardiomyocytes (10).

Cardiomyocytes isolated from a rodent heart are the most common model used to investigate the mechanism of many cardiovascular diseases development, including heart failure. In spite of many advantages of this model (accessibility, ethical reasons), there are some differences between the human and rat cardiomyocytes that have to be considered, among them shorter action potential, increased SERCA contribution to the relaxation, and about 6 times higher rate of physiological pacing are the most pivotal. In spite of a much higher rate of cardiomyocyte contractions in an intact heart, after isolation they are paced at much lower frequencies. It raises a question whether the parameters of contractions recorded in such non-physiological conditions reflect physiological ones.

The force-frequency relation in rats was examined in several papers, however most often not in the physiological range of frequencies. It has been shown in papillary rat muscle preparation decrease of the amplitude of contraction with increase in pacing frequency from 0.2 to 1 Hz, in the range far from the physiological condition (11).

Milani-Nejad et al. 2014 (12) have shown the augmentation of the contractions amplitude with increase in the frequency of pacing from 4 to 6 Hz, followed by a slight decrease at 7 Hz and a more prominent decrease at 8 Hz. However, the comparison of the contractile amplitude at the frequencies close to the physiological one was not referred to this parameter measured at lower frequencies. In contrast, Layland et al. (1996) (13) obtained force-frequency relationship in isolated rat ventricular trabeculae in a very wide range of frequencies (0.1-12 Hz), encompassing the physiological range. The relation was positive, peak force was achieved at 10 Hz and was 5 times higher than the force at 0.33 Hz. The results of these papers suggest that force-frequency relationships in the rat muscle preparation has a complex nature, depending on conditions associated with the experimental preparation and especially range of pacing frequencies used and it is rather positive, at least in the range of physiological frequencies.

Even more incomplete and often inconsistent results are obtained for isolated cardiomyocytes. Coutu et al. 2002 (14) have shown decrease in amplitude and relengthening time after increase in the pacing rate from 0.2 to 1.0 Hz, followed by an increase in these parameters with increasing the pacing rate from 2.0 and 4.0 Hz. Similarly, Ohtsuka et al. 2000 (15) have found negative force-frequency relationship in the range of frequencies 0.5-1 Hz and positive between 1 and 3 Hz. Fauconnier et al. (7) investigated the force-frequency relationship only for low frequencies (0.2-1 Hz) and found that it was positive. In contrast, Joulin et al. 2009 (16) have found a positive relation in the range from 0.5 to 2 Hz, while Liao et al. 2007 (17) have shown lack of difference in amplitude of contraction in cardiomyocyte paced at 0.5 and 2 Hz.

Generally, it may be concluded that shortening-frequency relationship in isolated rat cardiomyocytes exhibited a biphasic response to increasing stimulus frequency: rather negative for lower frequencies and positive or flat for higher (but still lower than physiological rate in rats) (4).

To address these disparities and lack of results for higher, more physiological frequencies we systematically investigated the amplitude and time-course of contractions in rat cardiomyocytes in physiological range of pacing frequencies and compared with those

obtained at low frequency, the most often used in *in vitro* experiments. We have shown that force frequency relationship is flat with insignificant decrease in amplitude at frequency higher than 6 Hz, which is in accordance with the results of Milani-Nejad et al. 2014 (12) obtained in muscle preparation. We did not find the difference between the amplitude of contraction at 1 Hz and at 6 Hz. Moreover, for the first time we have shown that relative contribution of contraction and relaxation phase to the whole cycle changes with increasing the pacing frequency, yet the difference did not exceed a few percent. In our model, increase in the pacing frequency to 4 Hz did not result in better reflection of physiological condition, because we did not find differences in respect to this parameter between 1 Hz and 4 Hz and the difference between 4 Hz and 6 Hz was still present. Thus, increase in the pacing frequency to 4 Hz in experiments in healthy cardiomyocytes is not necessary.

CONCLUSIONS

These results indicate that in adult cardiomyocytes isolated from a heart of a young healthy rat, the parameters of contraction-relaxation cycle at low pacing frequency surprisingly closely correspond with those measured at the frequency near the physiological heart rate (6 Hz): the amplitude of contraction did not differ and relative contribution of contraction and relaxation phases to whole cycle differed only by a few percent. The impact of pacing frequency on the contractile parameters is probably different in myocytes isolated from a failing, hypertrophied or ageing heart. Further research in this field is warranted.

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