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Role of adenosine triphosphate and protein kinase A in the force-frequency relationship in isolated rat cardiomyocytes

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Abstract: The physiological heart rate of rodents is around 4-6 Hz, although a stimulus frequency of 1 Hz is generally used in isolated cardiomyocytes to study changes in the contraction-relaxation cycle in cardiac muscle physiology and pathophysiology. Our study investigated the contraction parameters in isolated cardiomyocytes at 1, 2 and 4 Hz stimulation, and the roles of ATP and protein kinase A (PKA) in the force-frequency relationship in isolated cardiomyocytes. The contraction of the cell and intracellular Ca^{2+} changes were recorded simultaneously during cell stimulation by applying pulses of 6-8 V amplitude with frequencies of 1, 2 and 4 Hz. The increase in stimulus frequency caused a significant decrease in the percentage of shortening, relaxation times, slowing of the relaxation rate, and a significant increase in diastolic Ca^{2+} levels, but had no effect on the contraction rate and Ca^{2+} transients. Administration of ATP and N^6-benzoyladenosine-3'-5'-cyclic monophosphate (6-BNZ-cAMP) caused an increase in contraction amplitude and speed which were proportional to the stimulus frequency but had no effect on the relaxation times. The experimental results show that the force-stimulus frequency has a negative correlation in isolated myocytes and that energy metabolism and the β-adrenergic system may be responsible for this relationship.

Keywords: Ca^{2+}; adenosine triphosphate; protein kinase A; frequency; contraction

INTRODUCTION

Isolated adult cardiomyocytes are an important research tool for elucidating the mechanisms of physiological and pathophysiological pathways in cardiac muscle. The technique of cardiomyocyte isolation was first described more than 35 years ago. To date, numerous data, including cell electrophysiology, intracellular Ca^{2+} regulation, and cellular and subcellular physiology and pathology have been provided. Furthermore, the application of molecular biology techniques in isolated cardiomyocytes has provided a better understanding of protein expression, function and localization. In addition, the elaboration of cultured myocyte methods has enabled a wide range of toxicological studies, genetic manipulation in cardiomyocytes with viral-based gene transfer or gene silencing and the study of many pathophysiological conditions [1].

Despite the indisputable advantages of using isolated cardiomyocytes, some limitations of this model should be underlined. Isolated cardiomyocytes are deprived of their natural environment, which provides tight connectivity with other myocytes and components of the extracellular matrix. In this natural environment, apart from cardiomyocytes, the heart contains several physically and functionally interconnected cell types, mostly cardiac fibroblasts and endothelial cells [2]. Accordingly, from a "holistic" perspective, the heart has a complex multicellular network in which the extracellular matrix is held together by an interstitial compartment and encapsulated within a dense network of neurons [3,4]. Thus, while the contraction of cardiomyocytes *in vivo* interacts with forces generated in the extracellular matrix and other surrounding cardiomyocytes, isolated cardiomyocytes do not utilize environmental reinforcement [5]. Furthermore, as isolated cardiomyocytes remain separated from their natural environment, a shift in energy source occurs, leading to the utilization of glucose provided in the perfusion solution as an energy supply, whereas the energy required for cardiomyocytes

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in the living organism is obtained from *in vivo* fatty acids [6,7]. The heart must contract continuously (100,000 beats/day in a human) to ensure the circulation of sufficient blood (10 tons of blood/day in a human) to meet the perfusion and metabolic needs of the body [8]. Although the energy demand is huge, ATP (the main energy molecule) storage within the cardiomyocyte is minimal and insufficient to maintain sustained contraction. Therefore, a tight coupling with continuous and efficient ATP production is required for proper myocardial contraction [9]. Failure to produce sufficient amounts of ATP often results in cardiac contractile dysfunction and mechanical failure of the heart [8,10,11]. Furthermore, all of the aforementioned myocardial cells express receptors of sympathetic neurotransmitters [3,4] and owing to the capillary innervation of the heart, each of these cells is within short range of a neuronal network. The effects caused by sympathetic stimulation in the heart result from activation of β1-adrenoceptors, which are G protein-coupled receptors. The sympathetic neurotransmitter norepinephrine and other catecholamines, binds to β1 receptors and can activate adenylyl cyclase by causing a conformational change in excitatory G proteins. Activation of this enzyme then catalyzes the conversion of ATP to cAMP. Subsequently, this second messenger can trigger numerous other pathways, ion channels, transcription factors or enzymes. The most important enzyme activated by cAMP in the cardiovascular system is protein kinase A (PKA). Activated PKA phosphorylates contractile proteins involved in excitation-contraction coupling, such as L-type Ca^{2+} channels, ryanodine receptor (RyR), phospholamban (PLB) and troponins C, I and T. Sympathetic stimulation results in positive inotropic and lusitropic effects in myocytes [12]. *In vitro*, myocytes are deprived of the physiological regulation of the sympathetic system.

A second limitation in studies using isolated cardiomyocytes is the conditions of the bath environment. After isolation, cardiomyocytes are perfused with solutions that should mimic the physiological state in terms of ions and other substrate concentrations. The composition of the perfusion solution applied during the experiments varies among different laboratories. Although the temperature of the perfusion solutions was kept at physiological levels in some recent studies, cardiomyocytes were perfused at room temperature in many previous studies, resulting in much higher amplitudes and slower kinetics than observed in the physiological state [13].

Finally, the excitation frequency is also an important factor affecting the experimental results [14]. Contraction in isolated myocytes is achieved by applying electrical pulses to the myocytes in the bath solution by electrodes immersed in the solution. The frequency used in *in vitro* experiments is mostly 1 Hz, while the physiological heart rate in the rodent heart (the most common source of isolated cardiomyocytes) is about six (rat) or nine (mouse) times higher [15-17]. Low *in vitro* stimulation frequencies provide more stable recordings without the spontaneous contractions often observed with rapid application of stimulation frequencies. Recordings at low frequencies are preferred due to the establishment of a clear baseline between contractions, providing a more reliable analysis of the contraction signal, especially the time course. It is well known that an increase in stimulus frequency leads to a decrease in contraction and relaxation time [16,17]. Investigations into the effect of stimulation rate on contraction amplitude (force-frequency relationship) in isolated cardiomyocytes are rare and present inconsistent results [14,15].

The aim of this study was to investigate the effects of different stimulation frequencies on contraction amplitude, relaxation times and contraction velocity in the contraction-relaxation cycle of cardiomyocytes isolated from adult rat heart, and to investigate the changes that may occur in Ca^{2+} transients simultaneously with contraction-relaxation and to determine the role of energy metabolism and β-adrenergic system in these changes. Our results showed that isolated cardiomyocytes exhibit a negative force-frequency relationship due to insufficient energy metabolism and β-adrenergic system regulation. This situation can be compensated by adding ATP or PKA activator to the adaptation environment of ventricular myocytes.

MATERIALS AND METHODS

Ethics statement

Twelve three-month-old young adult male Wistar rats were used. The rats were obtained from the Experimental Animal Care and Production Unit of

Akdeniz University Faculty of Medicine with the approval of the Akdeniz University Animal Experiments Local Ethics Committee. The experimental animals were kept in a room at a temperature of 22°C±2°C and a 12-hour daylight/dark period with *ad libitum* access to commercial rat food and water without any restriction.

Myocyte isolation

From lightly anesthetized animals (50 mg/kg sodium pentobarbital), hearts were quickly removed and connected to the Langendorff system. Suspended hearts were first washed with a calcium-free perfusion solution containing 137 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.8 mM HEPES and 20 mM glucose, and gassed with 5% CO_2 and 95% O_2 to maintain pH 7.2 equilibrium, for 5 min. An enzyme mixture of collagenase (collagenase A, Worthington Biochemical Corp., USA) (0.7 mg/mL) and protease (0.06 mg/mL) prepared in the same solution was applied over the heart for 20-25 min and upon reaching appropriate consistency, the heart was placed in a small bowl. The left ventricle of the heart was separated and thinly sliced with scissors. The cells were then passed through a fine filter and subjected to several washes. Ca^{2+} adaptation of the cells in the medium was gradual [18].

Measurement of contractile parameters and intracellular free Ca^{2+} concentration

Isolated cardiomyocytes were incubated with fura2-AM (4 µM) for 15-20 min at room temperature, followed by incubation with Tyrode's solution (137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 11.8 mM Na-HEPES, 10 mM glucose, pH 7.35) in a cuvette with electrodes placed at both ends. Excitable cells were selected, and the amount of contraction produced by 1, 2 and 4 Hz with an amplitude of 6-8 V was recorded, while intracellular Ca^{2+} transients were simultaneously recorded by measuring fluorescence ratios centered at 510 nm by excitation at 340 and 380 nm (IonOptix LLC, Milton USA). In the contraction recordings, Ion wizard (IonOptix) program was used to calculate the percentage shortening rate (L/L_0); time from peak to 50% (RT_{50}), 75% (RT_{75}) and 90% (RT_{90}) decay of relaxation and contraction (−dL/dt) and relaxation (dL/dt) rates. In Ca^{2+} transients, the maximum value of $[Ca^{2+}]_i$ signals measured by subtracting from the basal value ($\Delta FFI_{340/380}$) and time to 37% decay of maximum value by applying an exponential function (τ_{decay}) were measured and compared.

Effects of ATP and PKA on contraction

To determine the effects of energy metabolism and the β-Adrenergic receptor (β-AR) system on the changes that occur with increasing excitation frequency, cells were incubated with 4 µM fura-2-acetoxymethyl ester (fura-2 AM) simultaneously with 1 mM ATP or 2 nM ATP inhibitor carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) or 100 µM PKA activator N^6-Benzoyladenosine-3'-5'-cyclic monophosphate (6-BNZ-cAMP) or 1 µM PKA inhibitor KT5720 for 30 mins before the contraction protocol was applied. Contraction and Ca^{2+} transient recordings and analysis were performed as described above.

Statistical analysis

Statistical evaluation was performed using the SPSS package program. Results are given as the mean±SEM. Normality test was applied for each variable. The parametric one-way analysis of variance (ANOVA) followed by Tukey's post hoc test were used for data that show the normal distribution. $P<0.05$ was considered statistically significant.

RESULTS

Contraction and intracellular Ca^{2+}-related parameters

Contraction-relaxation phases and intracellular Ca^{2+} transients

To determine the force-frequency relationship of isolated cardiomyocytes, contraction responses were recorded at 1, 2 and 4 Hz stimulus frequencies (Fig. 1A). When the ratio of the shortening of the cardiomyocyte length to the initial length (fractional shortening) was examined, it was observed that the fractional shortening in response to 1 and 2 Hz stimuli varied at the rates of 9.96±0.59% and 9.80±0.49%, respectively, with no statistical difference between these frequencies.

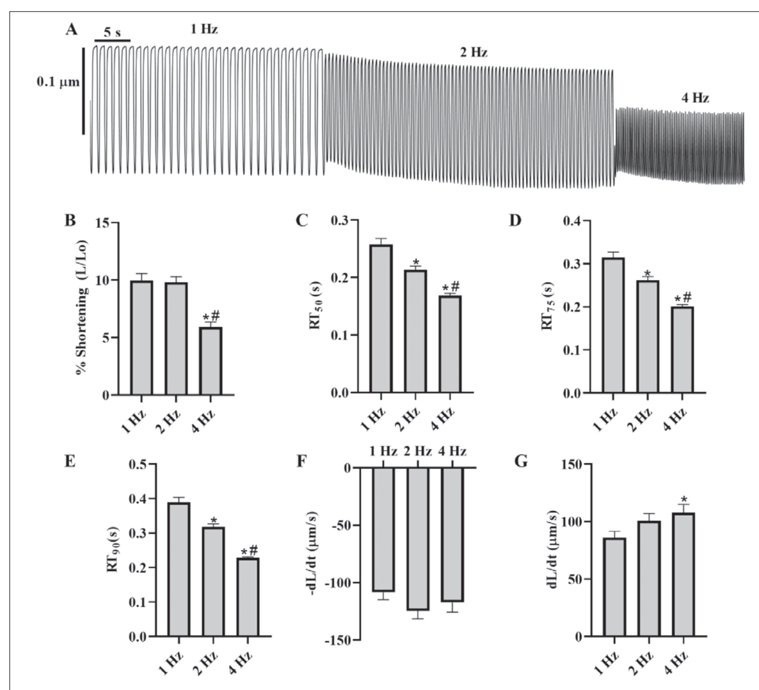

Fig. 1. Contraction responses obtained in cardiomyocytes stimulated with an electric field at frequencies of 1, 2 and 4 Hz. **A** – Myocyte contraction pattern for 1, 2 and 4 Hz stimulation frequencies. **B** – Myocyte shortening rate (fractional shortening). **C** – Time from peak to 50% decay of relaxation. **D** – Time from peak to 75% decay of relaxation. **E** – Time from peak to 90% decay of relaxation. **F** – Contraction rate. **G** – Relaxation rate. Values are given as the mean±SEM; n=56, 56, 50 cells from 5 rats 1 Hz, 2 Hz and 4 Hz per CON respectively, difference compared to 1 Hz, *P<0.05; difference compared to 2 Hz, #P<0.05.

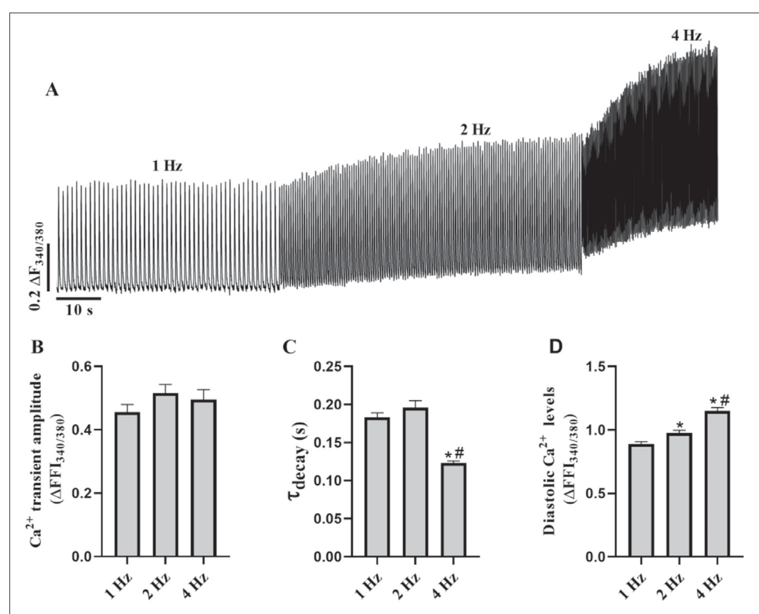

Fig. 2. Ca^{2+} transients obtained from cardiomyocytes stimulated with electric field pulses with frequencies of 1, 2 and 4 Hz. **A** – Myocyte Ca^{2+} transient pattern for stimuli with frequencies of 1, 2 and 4 Hz. **B** – Amplitude values of Ca^{2+} transients. **C** – Time constant of Ca^{2+} transients. Values are given as the mean±SEM; n=56, 56, 50 cells from 5 rats 1 Hz, 2 Hz and 4 Hz per CON respectively; difference from 1 Hz, *P<0.05; difference compared to 2 Hz, #P<0.05.

However, when the stimulus frequency was increased to 4 Hz, the fractional shortening decreased to 5.91±0.43% (Fig. 1B). On the other hand, when the relaxation times were analyzed with increasing frequency, they were observed to be shortened at all analysis points (RT50, RT75 and RT90) (Fig. 1C, D, E). After 1 and 2 Hz stimulation, there was no significant difference in fractional shortening, but the increase in frequency caused a shortening in relaxation time. At 4 Hz, the relaxation times may also be shorter as the fractional shortening rate decreased by about 50%. Analysis of the contraction and relaxation rates is crucial to better understand the variation in relaxation times. While the change in stimulation frequency did not show a significant impact on the contraction rate, the relaxation rate increased in parallel with the frequency (Fig. 1F, G). These results suggest that in excitation-contraction coupling, stimulation at low frequencies may be more effective on the relaxation times, whereas high frequencies may be more effective on the relaxation rate.

To reveal the relationship between Ca^{2+} regulation and the mechanical responses induced by changes in stimulation frequency, Ca^{2+} transients were recorded in fura2-AM-loaded cardiomyocytes to determine the amount and the kinetics of the Ca^{2+} released into and reabsorbed from the intracellular medium simultaneously with contraction (Fig. 2A). Assessment of frequency-dependent Ca^{2+} transient amplitudes in the contraction-relaxation cycle demonstrated that the stimulation frequency did not cause a significant change in amplitude (Fig. 2B), but the transient basal level value indicating diastolic Ca^{2+} increased significantly at 4 Hz stimulation (1 Hz: 0.89±0.01; 2 Hz: 0.97±0.2; 4 Hz: 0.149±0.02).

The time to reach 37% of the maximum value of Ca^{2+} transients (τ_{decay}) recorded in isolated cardiomyocytes showed that although there was no significant difference in Ca^{2+} transient amplitudes, τ_{decay} was

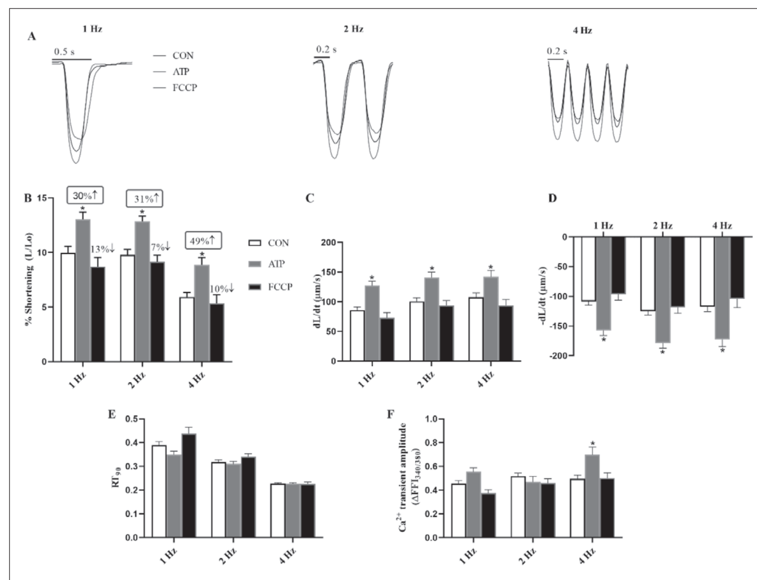

Fig. 3. Effects of 1 mM ATP and 2 nM FCCP on the contraction and Ca^{2+} transients in cardiomyocytes stimulated with 1, 2 and 4 Hz frequency electric field stimuli. **A** – Myocyte contraction patterns of the groups for 1, 2 and 4 Hz stimulation frequencies. **B** – Myocyte shortening rate (fractional shortening). In ATP application, the % shortening rates increased 30% for 1 Hz, 31% for 2 Hz and 49% for 4 Hz compared to CON. **C** – Contraction rate. **D** – Relaxation rate. **E** – Time from peak to 90% decay of relaxation. **F** – Amplitude values of Ca^{2+} transients. Values are given as the mean±SEM; n = 56, 56, 50 cells from 5 rats 1 Hz, 2 Hz and 4 Hz per CON respectively; n=25, 25, 20 cells from 3 rats 1 Hz, 2 Hz and 4 Hz ATP respectively; n=31, 31, 20 cells from 3 rats 1 Hz, 2 Hz and 4 Hz FCCP respectively, difference compared to CON group, $^{*}P<0.05$.

considerably shortened at 4 Hz stimulation (Fig. 2C). This is because higher stimulation frequencies lead to a marked increase in the diastolic Ca^{2+} level of ventricular myocytes (Fig. 2D). These results suggest that isolated cardiomyocytes are incapable of complete removal of Ca^{2+} from the intracellular environment during stimulation at high frequencies when changes at the basal level are considered.

Contraction-relaxation phases of ATP and their impact on Ca^{2+} transients

The influence of ATP, which has a primary role in contraction, on the changes of fractional shortening, relaxation times, velocity parameters and Ca^{2+} transients, which are shown by the analysis of contraction-relaxation phases in isolated rat cardiomyocytes, was investigated. Contraction and simultaneous Ca^{2+} transient recordings were obtained from myocytes incubated with fura2-AM, ATP and FCCP (Fig. 3A). Compared to matching controls, the ATP-induced increase in fractional contraction at 1 and 2 Hz stimulation frequencies was significant (30% and 31%, respectively). In contrast, at higher stimulation with 4 Hz, ATP significantly compensated for the 50% reduction seen in fractional shortening relative to lower frequencies and elicited a 49% increase with respect to the matching control (Fig. 3B). ATP application increased the fractional shortening by about 30% at 1 and 2 Hz stimulation frequencies, and by 49% at 4 Hz stimulation. This result suggests that intracellular ATP is insufficiently regulated in isolated cardiomyocytes during high-frequency stimulation. In addition, ATP blockade was provided with FCCP application, and it was confirmed that ATP was effective on contraction. It was also observed that ATP had positive inotropic and lusitropic effects at three different frequencies during contraction-relaxation phases (Fig. 3C, D). The relaxation phases shortened with increasing stimulation frequency. ATP and FCCP treatments had no impact on the changes in relaxation times (Fig. 3E).

When recordings of Ca^{2+} transients taken simultaneously with contraction were analyzed, no significant difference was found in Ca^{2+} amplitude at 1, 2 and 4 Hz stimulation, but ATP application triggered an increase in Ca^{2+} amplitude at high frequency stimulation (Fig. 3F). When τ_{decay} of Ca^{2+} transients were analyzed, no difference was found between the τ_{decay} values obtained in the absence and after ATP treatment in isolated cardiomyocytes. On the other hand, diastolic Ca^{2+} levels did not change at any stimulus frequency after ATP administration.

Contraction-relaxation phases of PKA activation and their impact on Ca^{2+} transients

To determine whether the results obtained in the contraction-relaxation phases of isolated cardiomyocytes were related to the lack of β-AR regulation in isolated cardiomyocytes, cells were incubated with 6-Bnz-cAMP or KT-5720 simultaneously with fura2-AM

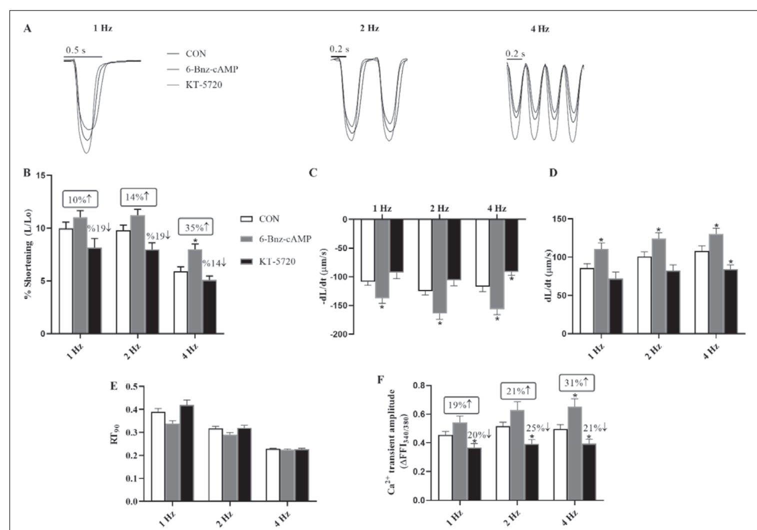

Fig. 4. Effects of 100 μM 6-Bnz-cAMP and 1 μM KT-5720 on contraction and Ca^{2+} transients in cardiomyocytes stimulated with 1**,** 2 and 4 Hz frequency. **A**– Myocyte contraction patterns of the groups for 1, 2 and 4 Hz stimulation frequencies. **B** – Myocyte shortening rate (fractional shortening) In 6-Bnz-cAMP application, the % shortening rates increased 10% for 1 Hz, 14% for 2 Hz and 35% for 4 Hz compared to CON. **C** – Contraction rate. **D** – Relaxation rate. **E** – Time from peak to 90% decay of relaxation. **F** – Amplitude values of Ca^{2+} transients. In 6-Bnz-cAMP application, Ca transient amplitudes increased by 19% for 1 Hz, 21% for 2 Hz, and 31% for 4 Hz compared to CON. Values are given as the mean±SEM; n=56, 56, 50 cells from 5 rats 1 Hz, 2 Hz and 4 Hz per CON respectively; n=37, 37, 33 cells from 4 rats 1 Hz, 2 Hz and 4 Hz 6-Bnz-cAMP respectively; n=32, 32, 29 cells from 4 rats 1 Hz, 2 Hz and 4 Hz KT-5720, respectively, difference compared to the CON group, *P<0.05.

for 30 min, and contraction and Ca^{2+} transients were recorded for 1, 2 and 4 Hz (Fig. 4.A). As in ATP application, at 1 and 2 Hz stimulus frequencies 6-Bnz-cAMP and KT-5720 applications showed a similar trend (at 1 Hz, 6-Bnz-cAMP caused 10% increase; at 2 Hz, 6-Bnz-cAMP application caused a 14% increase) although they could not reach a significant level. In contrast, 6-Bnz-cAMP induced a 35% and statistically significant increase in contraction at high-frequency stimulation of 4 Hz (Fig. 4B). These results suggest that the lack of regulation of the β-adrenergic system in isolated cardiomyocytes can lead to a decrease in contraction amplitude during stimulation at high frequencies. In addition, positive inotropic and lusitropic effects of 6-Bnz-cAMP were also observed at the three different frequencies during contraction-relaxation phases (Fig. 4C, D). Although there was a shortening in relaxation times with the increase in stimulus frequency, 6-Bnz-cAMP administration did not have a significant effect (Fig. 4E).

Although there was no stimulus frequency-dependent change in Ca^{2+} transients in isolated myocytes at baseline medium, 6-Bnz-cAMP, a PKA activator (an important mediator of Ca^{2+} regulation), caused an increase in Ca^{2+} transients proportional to the stimulus frequency, although it was significant only at 4 Hz (Fig. 4F). When the decay times of Ca^{2+} transients were examined, no difference was found between the τ_{decay} values obtained in isolated control cardiomyocytes and the τ_{decay} values obtained after 6-Bnz-cAMP treatment. In addition, the increase in diastolic Ca^{2+} levels in CON myocytes at 4 Hz stimulation was significantly reduced by 6-Bnz-cAMP treatment, while this was not observed for 1 and 2 Hz (for 1 Hz, CON: 0.890±0.018 and 6-Bnz-cAMP: 0.880±0.025; for 2 Hz, CON: 0.976±0.021 and 6-Bnz-cAMP: 0.987±0.028; for 4 Hz, CON: 0.149±0.02 and 6-Bnz-cAMP: 0.125±0.031).

DISCUSSION

In the present study, the effects of stimulation frequency on the fractional shortening rates of contractions, relaxation times, contraction-relaxation rates and simultaneous Ca^{2+} transients recorded in isolated cardiomyocytes at the stimulation frequency range most frequently used in *in vitro* experiments (1, 2, and 4 Hz) were investigated. In addition, the reasons for the observed changes in contraction parameters due to the increase in stimulus frequency were examined. The amplitude of fractional shortening, a measure of contraction force, decreased significantly at 4 Hz stimulation and the duration of relaxation phases decreased gradually with increasing stimulation frequency. In addition, the increase in frequency caused no change in the contraction rate, whereas a gradual increase in the relaxation rate occurred. Furthermore, the increase in stimulus frequency caused a decrease in the time to reach 37% of the maximum value of Ca^{2+} transients. These results suggest that in cardiomyocytes isolated from the healthy heart of a young rat there may be differences in the contraction-relaxation cycle between the low stimulus frequency (1 Hz) and the

frequency close to the physiological heart rate (4 Hz). The reasons for these differences were investigated and it was concluded that there may be a decrease in the contraction amplitude because of the inability of isolated myocytes to produce ATP, the basic molecule of energy metabolism, in a sufficient amount *in vitro*, and the lack of regulation of PKA, the main mediator of the β-adrenergic system, with increasing stimulation frequency.

In the heart muscle, apart from the Frank-Starling mechanism, which is the heart's ability to change the force of contraction and hence stroke volume in response to changes in venous return, the strength of contraction is regulated by the heart rate. This regulation provides an intrinsic mechanism that is essential for cardiac contractile function to respond immediately to rapid changes in body requirements. In response to an increase in heart rate, Na^+ ion accumulation occurs in cardiomyocytes due to insufficient activity of Na^+/K^+ ATPase, followed by a decrease in Ca^{2+} excretion into the extracellular medium by the Na^+/Ca^{2+} exchanger. The increase in intracellular Ca^{2+} leads to increased activation of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) and increased sarcoplasmic reticulum (SR) Ca^{2+} content. This results in high Ca^{2+} release from the SR and increased force of contraction. This is accompanied by faster relaxation and ensures proper ventricular filling at high heart rates [19]. The positive force-frequency relationship occurs in many mammalian hearts, including humans. However, a negative force-frequency relationship has been observed in heart failure due to abnormal Ca^{2+} utilization [20].

Myocytes isolated from rodent hearts are the most common model used to investigate the mechanism of development of many cardiovascular diseases, including heart failure. Despite the many advantages of this model (accessibility, ethical reasons), there are some important differences between human and rat cardiomyocytes that need to be considered, such as action potential durations, the contribution of SERCA to relaxation and physiological rates of excitation. In the whole heart, cardiomyocytes contract at much higher excitation frequencies, but after isolation they can respond to stimuli at lower frequencies. Therefore, whether contraction parameters recorded under non-physiologic conditions reflect the physiological environment is a crucial issue that needs to be addressed.

There have been studies examining the force-frequency relationship in cardiomyocytes isolated from rats, but the results obtained are generally inconsistent. In a study of cardiac papillary muscle, it was observed that the contraction amplitude decreased as stimulus frequency increased from 0.2 to 1 Hz [21]. In another study, it was shown that the contraction amplitude increased when the stimulus frequency was increased from 4 to 6 Hz, followed by a slight decrease at 7 Hz and a pronounced decrease at 8 Hz in the trabecular structure isolated from the right ventricle [22]. In contrast, a positive force-frequency relationship was recorded in isolated rat ventricular trabeculae over a very wide frequency range (0.1-12 Hz), including the physiological range [23]. The results of these studies show that the force-frequency relationships in rat cardiac muscle have a complex process that depends on the experimental conditions and especially the stimulus frequency range used. Even more incomplete and often inconsistent results have been obtained for isolated cardiomyocytes. A decrease in contraction amplitude and relaxation times after increasing the stimulus frequency from 0.2 to 1.0 Hz, followed by an increase in these parameters by increasing the stimulus frequency to 2 and 4 Hz was obtained [24]. Similarly, a negative force-frequency relationship in the frequency range of 0.5-1 Hz and a positive force-frequency relationship between 1 and 3 Hz were observed [25]. Investigation of the force-frequency relationship only for low frequencies (0.2-1 Hz) revealed a positive correlation [17]. In contrast, a positive relationship between 0.5 to 2 Hz was shown [26], but there was no difference in contraction amplitude in cardiomyocytes stimulated at 0.5 and 2 Hz [27]. On the other hand, there was no difference in contraction amplitudes in the range of 1-6 Hz, and only a partial change in contraction and relaxation times, but this change was not significant [28]. In our study, we examined contraction parameters at 1, 2 and 4 Hz stimulation frequencies and showed that there was no difference in 1 and 2 Hz stimulation, but there was a decrease in contraction amplitude, an increase in relaxation rate and a gradual decrease in relaxation times at 4 Hz stimulation. The diversity of the tissues or cells studied, the lack of regulatory systems because of removal of tissues or cells from their physiological environment, the temperature of the working environment and the difference in the contents of the solution used in the isolation and adaptation process stand out

as an important problem in the comparison and interpretation of *in vitro* studies. The solution contents in isolation and adaptation environments are of great importance in terms of obtaining results like physiological responses and interpreting these results correctly.

Conversely, heart rate acutely regulates the contractile state, and an increased heart rate in the mammalian myocardium has been shown to increase contractile activity in a wide variety of animal species [19,29,30]. With the increase in heart rate, a large amount of energy is needed for a strong contraction, but the amount of ATP stored in the cardiomyocyte is minimal and not enough to maintain sustained contraction. Therefore, a continuous and efficient production of ATP is required for proper myocardial contraction [31]. However, extracellular ATP has been shown to have both negative and positive inotropic effects on contraction [32]. Most early reports described negative inotropic effects after the administration of ATP in mammalian hearts, especially in the atrium [33-36]. Subsequent studies on guinea pig and rat atria revealed the positive inotropic effects of ATP following a transient and rapid decline [37,38]. In our study, a decrease in contraction amplitude was observed at 4 Hz stimulation frequency in isolated cardiomyocytes separated from their physiological environment in which glucose was used as the energy source, and this decrease was significantly reversed by ATP application and the contraction amplitude was close to the contraction rate obtained at 1 Hz stimulation. Our results suggest that the positive inotropic effect disappears in isolated cardiomyocytes due to the inadequate production of ATP at high frequencies (≥4 Hz).

The autonomic nervous system, another essential system in the regulation of the contractile function of the heart, continuously controls cardiac performance through cardiac sympathetic and parasympathetic neurons to match blood output to the perfusion demand of the organism, both during daily activities and in response to stressors [39]. Autonomic activation alters not only heart rate, conduction and hemodynamics, but also the cellular and subcellular properties of individual myocytes [40]. The contractile force of the heart is determined by the increased degree of binding between myosin and actin filaments, which depends on the Ca^{2+} concentration in the cardiomyocyte cytosol. Stimulation of the sympathetic nervous system causes an increase in intracellular Ca^{2+} levels, resulting in an increase in contraction in the ventricles and atrium [41]. PKA activated by β-AR stimulation causes an increase in Ca^{2+} influx through L-type Ca^{2+} channel phosphorylation. This increase leads to an increase in RyR phosphorylation and a subsequent increase in Ca^{2+} released from the SR, i.e., an increase in Ca^{2+} transient amplitude, thereby providing a large amount of Ca^{2+} for sarcomere contraction in systole. In conclusion, the inotropic effects of PKA are mediated by L-type Ca^{2+} channels and RyR. On the other hand, phosphorylation of troponin I and MyBP-C by PKA decreases the sensitivity of sarcomeric myofilaments to Ca^{2+}, leading to rapid dissociation of myofilaments from Ca^{2+}. Phosphorylation of the PLB protein on SERCA by PKA also increases SERCA activity, allowing rapid clearance of Ca^{2+} from the cytosol, and all these processes mediate the lusitropic effects of PKA [42]. In our study, incubation of isolated myocytes with PKA activator significantly improved the decrease in contraction amplitude caused by the increase in stimulation frequency. In addition, an increase in Ca^{2+} transient amplitude was also observed in proportion to the increase in stimulation. These results suggest that isolated cardiomyocytes outside of their physiological environment show a negative force-frequency relationship in the event of an increase in stimulus frequency because they are deprived of β-adrenergic system regulation.

CONCLUSIONS

We demonstrated that the contraction-relaxation cycle parameters at the low-to-moderate stimulus frequency (1-2 Hz) used in *in vitro* experiments differ from those obtained at a frequency close to the physiological beat frequency (4 Hz) in cardiomyocytes isolated from the heart of a young, healthy rat. These differences are due to poorly regulated energy metabolism of isolated cardiomyocytes at high stimulation frequencies and the lack of control of the β-adrenergic system. Therefore, we can conclude that physiological stimulation frequencies should be used instead of 1 Hz stimulation in experimental studies with isolated myocytes, which is an often-debated issue. However, since the isolated myocytes are separated from their supporting environments, they cannot be expected to fully function as in the intact heart at high frequencies. Another option

is to optimize the parameters that limit *in vitro* studies in obtaining results representative of physiological responses in these experiments. For this reason, it is likely that the addition of ATP and PKA to the adaptation solutions can prevent the negative force-frequency relationship at high frequencies to support the energy metabolism and the β-AR system, which are two important systems regulating the contraction force *in vitro*.

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Conflict of interest disclosure: The authors declare no conflict of interest.

Data availability: All data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Ozturk%20et%20al_8232_Data%20Report.pdf

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