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Compact cell image projector: application to study the relationship between stimulus interval and contraction amplitude in isolated rat cardiomyocytes

Projetor compacto de imagem celular: aplicação ao estudo da relação entre intervalo entre estímulos e amplitude de contração em cardiomiócitos isolados de rato

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Abstract
Cardiac inotropy depends, among other factors, on the interval between contractions. In this study, we developed instrumentation for cell shortening recording, which was used to investigate the influence of stimulatory rhythm on contraction amplitude of isolated rat ventricular myocytes. Peak cell shortening amplitude was recorded during electric stimulation at the average rate of 0.5 Hz with different stimulatory patterns: regular and pseudo-random rhythms, as well as double pulse stimulation. Cells were perfused at 23 ºC with modified Tyrode's solution with or without 10 nM isoproterenol (ISO). The main advantages of the developed microscopy system were its relatively low cost (~US$ 1,000.00), small size (150 × 170 × 300 mm), and absence of detectable optic distortions. We observed that average contraction amplitude was similar for all stimulatory patterns, in the absence and presence of ISO ($p > 0.05$), although the amplitude of individual contractions was highly dependent on the previous interval, and was significantly increased by ISO ($p < 0.05$). With the double pulse patterns, the amplitude ratio of contractions following the shorter and the longer intervals was ~0.55. ISO positive inotropic effect was more prominent for contractions after short intervals, which increased the ratio to ~0.80. This might be explained by acceleration of the recovery of sarcoplasmic reticulum Ca^{2+} release channels from the adapted state, possibly by protein kinase A-dependent phosphorylation, which would result in enhanced systolic Ca^{2+} release.
Keywords: Adrenergic stimulation, Biomedical instrumentation, Cardiac inotropy, Cardiomyocytes, Heart rate and rhythm, Image projector.

Resumo
O inotropismo cardíaco depende de inúmeros fatores, entre eles o intervalo entre contrações. Neste trabalho, desenvolvemos instrumentação para registro de encurtamento celular e investigamos a influência do ritmo estimulatório sobre a atividade contrátil de miócitos ventriculares isolados de rato. A amplitude do encurtamento celular foi registrada durante estimulação elétrica à freqüência média de 0,5 Hz, com ritmo regular, ritmo pseudo-aleatório e pulsos duplos. Os miócitos foram perfundidos a 23 ºC com solução de Tyrode modificada contendo ou não 10 nM de isoproterenol (ISO). O sistema de microscopia desenvolvido é de custo relativo baixo (~US$ 1.000,00), dimensões reduzidas (150 × 170 × 300 mm) e apresenta boa qualidade óptica (sem distorções ou paralaxe detectáveis). Observamos que a amplitude média das contrações foi semelhante em todos os ritmos estimulatórios na ausência e presença de ISO ($p > 0,05$), embora a amplitude de contrações individuais fosse dependente do intervalo precedente, e ISO tenha causado aumento da amplitude média das contrações ($p < 0,05$). Nos padrões com pulso duplo, a razão de amplitude das contrações que seguem o menor e o maior intervalo foi ~0,55. O efeito inotrópico positivo de ISO foi mais pronunciado para contrações após intervalos curtos, o que levou a razão para ~0,80. Isto poderia ser explicado por aceleração da recuperação dos canais de liberação de Ca^{2+} do retículo sarcoplasmático do estado adaptado, causada possivelmente por fosforilação pela proteína quinase A, o que aumentaria a quantidade de Ca^{2+} liberada durante a sístole.
Palavras-chave: *Cardiomiócitos, Estimulação adrenérgica, Frequência e ritmo cardíaco, Inotropismo cardíaco, Instrumentação biomédica, Projetor de imagens.*

Introduction

More than a century has elapsed since Bowditch (1871) first described that the force developed by the cardiac muscle is dependent on the interval between contractions. From then on, many studies have contributed to improve our understanding of the mechanisms involved in this phenomenon (Hadju, 1953; Hoffman & Kelly, 1959; Koch-Weser & Blinks, 1963; Bassani *et al*., 1990; Maier *et al*., 2000). However, many aspects of this relationship still remain to be elucidated, such as the reciprocal regulation of cardiac spontaneous frequency and developed force, and the effects of β-adrenergic stimulation on the force-frequency relationship (Lakatta, 2004). The dependence of the force developed by the myocardium on the interval between contractions can be easily demonstrated in many preparations using a great variety of stimulation protocols (e.g. post-stimulation potentiation, post-rest potentiation or decay of twitch amplitude, force-frequency relationship). Frequency and interval between contractions are often taken as interchangeable (or inverse functions), but this is true only if the time-distribution of the interval between contractions is regular, that is, for a given time of observation, the interval between consecutive contractions is constant. In the more general case, probably closer to the heart rhythm *in vivo*, intervals are far from constant, so that heart rate and developed force over a given period should be considered as average values. *In vivo* beat-to-beat intervals are subject to considerable variation (e.g., physiologic respiratory sinus arrhythmia, Yasuma & Hayano, 2004), which has been correlated to the degree of autonomic modulation of chronotropism (Aubert & Ramaekers, 1999; Stein & Kleiger, 1999; Lewis, 2005).

If force is somehow dependent on the preceding (one or more) intervals, any change in rhythm will cause change in contractile performance. Which could be considered as the main determinant of the average force developed by the cardiac muscle: rate or rhythm? In the present work, we tested the hypothesis that average frequency determines the average force in isolated rat cardiac myocytes, but that instant interval changes may significantly affect contraction amplitude, under both control condition and inotropic stimulation.

To accomplish this, we have developed a simple, portable and low cost device, which we call Compact Cell Image Projector (CCIP), to amplify and project images of single isolated cardiac cells on a CCD camera. The device may be used for measuring cell dimensions, as well as cell shortening during functional experiments. Special stimulation protocols were also developed using the Labview™ system, aiming at producing different stimulation patterns at a constant average frequency.

Methods

Compact Cell Image Projector

As in an inverted microscope, the light source (Super Bright LED, Jumbo, 5,000 mcd, 1.85 V, 20 mA, Archer #276-086 – Figures 1A and 1B) in the CCIP is above the stage, pointing down on the vertical line formed by the objective (DPlan 40, Olympus, Tokyo) axis, so that light passing through the sample produces an image that is reflected by the internal mirror (or prism) to a CCD video camera (ICD-31, Ikegami Tsushinki Co., Tokyo). A perfusion chamber or any other specimen holder with a transparent bottom (e.g., coverslip) is placed on the x-y stage, which can be moved in two directions, allowing the image of a particular cell in the sample to be displayed on the video monitor (Figure1A). For the present experiments, the distance between the objective base and the CCD sensor (tube length) was set at 116 mm, so that an object measuring 120 µm would occupy all the longitudinal length of the sensor. Total amplification in the optical system (1,740×) was calculated as the product of optical amplification and the ratio between monitor and sensor dimensions. Images were recorded on a videocassette recorder (VCR) in order to allow off-line processing.

A standard graticule (10 µm precision, Carl Zeiss, Göttingen) was positioned on the stage, so that its image would be displayed in a given quadrant of the video monitor. In each quadrant, a distance Δx (10 µm) was measured using a video-edge detector (VED), of which the voltage output (E_{VED}) is linearly related to displacement of its sensor on a video screen with a precision of ~0.02 µm (Steadman *et al*., 1988). This test was designed to detect possible parallax-like distortions introduced by CCIP optics. The same graticule was used for linearity analysis, by varying Δx from 10 to 40 µm in 10 µm steps, and relating E_{VED} to Δx. Five replicates were made for each measurement.

Isolated Rat Ventricular Myocytes

Ventricular myocytes were isolated enzymatically from adult male Wistar rats by coronary perfusion with collagenase type I (0.5-0.7 mg/ml, Worthington Biochem. Corp., Lakewood, NJ), followed by mechanical dissociation, as described by Bassani & Bassani (2002). The protocol was approved by the institutional

Ethics Committee for Animal Experimentation (Proc. #952-1).

Cells (used within 8 h from isolation) were plated onto a perfusion chamber (Centro de Engenharia Biomédica, patent #PI0302.403.2) especially designed to fit on the stage of the CCIP. Cells were perfused with modified Tyrode's solution (NT, with the following composition (mM): 140 NaCl; 6 KCl; 1.5 $MgCl_2$; 5 4-(2-hydroxyethyl)-l-piperazine-ethanesulfonic acid (HEPES); 11.1 glucose; 1 $CaCl_2$; pH 7.4 at 23 ºC, adjusted with NaOH) and field stimulated at 0.5 Hz with bipolar voltage pulses (amplitude 1.2× threshold, 2 ms duration).

Stimulation Protocols and Experimental Procedure

Using the Labview™ software (Labview 7.1, National Instruments Co., Austin, TX), we programmed five stimulation protocols, and used voltage pulses to trigger the electrical stimulator (Figure 1A), to which the stimulation electrodes were connected. Trigger pulse time distributions were designed to produce: a) a basic rhythm (BR), in which stimulus distribution was regular (2 s interval); b) a pseudo-random stimulus distribution (RD), with time intervals of 2, 1, 1.5, 2.5 and 3 s; two patterns of double pulses: c) DP1000, in which stimuli were separated by alternating intervals of 1 and 3 s; and, d) DP700, in which stimuli were separated by alternating intervals of 0.7 and 3.3 s (Figure 2). Thus, cells were stimulated at the constant average frequency of 0.5 Hz, but with different patterns (rhythms).

After 5 min stimulation with a given protocol to attain steady-state contraction amplitude, cell length was measured during 10 successive contractions, and the average peak shortening during a twitch (ΔCL) was computed and expressed as percent of the resting cell length (% RCL), which was measured directly on the video monitor. Maximum $((dL/dt)_{max})$ and minimum $((dL/dt)_{min})$ time derivatives of cell length change during contraction and relaxation, respectively, and the half-time for cell relaxation ($t_{1/2\text{-}rel}$) were also estimated in cells paced with protocols DP1000 and DP700. Measurements were repeated 5 min after addition of 10 nM isoproterenol (ISO, Sigma Co., S. Louis, MO), to stimulate β-adrenoceptors. All experiments were carried out at room temperature (23 ± 2 ºC).

Statistical Analysis

Data are presented as mean ± standard error (SEM).

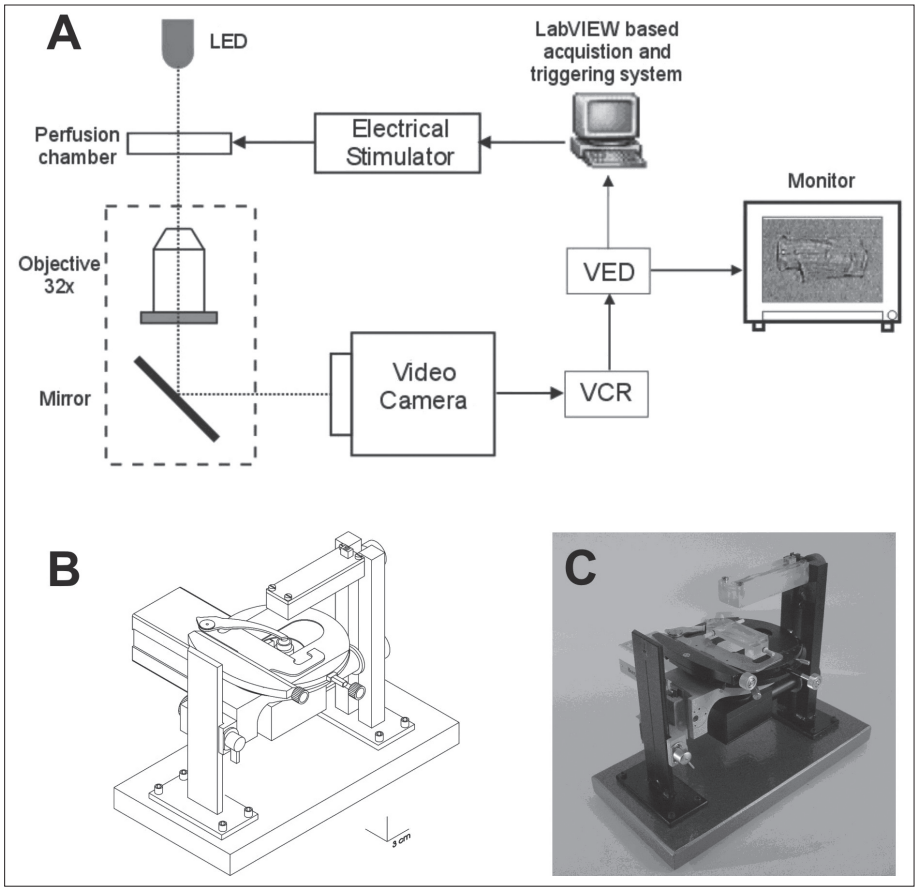

Figure 1. A) Instrumentation used for the present experiments: video-edge detector (VED); video-cassette recorder (VCR); the compact cell image projector (CCIP) is delimited by a dashed line. B) and C) CCIP.

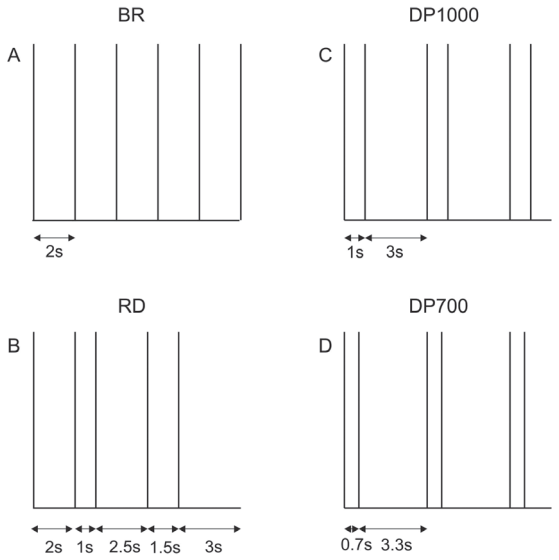

Figure 2. Trigger pulse time distribution for the several stimulatory patterns: A) Basic rhythm (BR); B) Pseudo-random distribution (RD). Double pulses protocols DP1000 (C) and DP700 (D). Intervals between pulses (in s) are indicated.

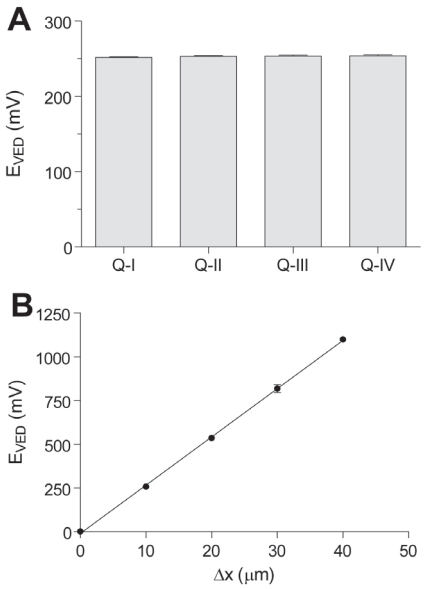

Figure 3. CCIP tests. A) E_{VED} for a 10 μm displacement performed at each of the quadrants of the video monitor. B) Relationship between E_{VED} and displacement (Δx). Means and SEM of 5 replicates are presented. Best fitting equation: E_{VED} = (27.59 mV/μm · Δx) - 10.72 mV.

One- or two-way analysis of variance followed by Bonferroni t test was used to investigate the influence of the stimulation pattern and/or β-adrenergic stimulation on the contraction and relaxation parameters, as well as for the parallax test. For the CCIP linearity test, linear regression was followed by runs test for departure from linearity (Prism 4.0, GraphPad Software Inc, San Diego, CA). Statistical significance was considered to occur when $p < 0.05$.

Results

CCIP: Parallax and Linearity Tests

As seen in Figure 3A, E_{VED} in response to a 10 μm displacement was similar for all quadrants of the video monitor on which the measurement was made ($p = 0.453$, one-way analysis of variance), which indicates the absence of significant parallax distortions. Linearity between Δx and E_{VED} was significant ($R^2 = 0.994$; $p = 0.50$ for the runs test, Figure 3B).

Influence of Stimulation Interval on Twitch Amplitude

The effect of the stimulatory rhythm on ΔCL is depicted in Figure 4. The average ΔCL (as % of RCL) was not statistically different among the four stimulation protocols (7.3 ± 0.7 (n = 20) for BR; 6.7 ± 0.8 (n = 13) for DR; 7.7 ± 2.0 (n = 4) for DP700; and 7.2 ± 0.8 (n = 16) for DP1000, $p = 0.936$), in which the average frequency was the same (0.5 Hz), although amplitude of individual twitches varied within and among the different rhythms (i.e., there was a positive relationship between twitch amplitude and duration of the preceding interval). Thus it seems that frequency, rather than rhythm, is the main determinant of the average peak cell shortening.

Influence of β-Adrenergic Stimulation on Interval-Dependence of Twitch Amplitude

The β-adrenoceptor agonist ISO (10 nM), a well-known positive inotropic agent, markedly increased the average twitch amplitude in cells paced at the BR (7.3 ± 0.7 and 11.9 ± 1.2% of RCL in the absence and presence of ISO, n = 20 and 12, respectively; Figure 5A). The same was observed when cells were paced with the RD protocol (6.7 ± 0.8 and 11.8 ± 1.9% of RCL in the absence and presence of ISO, n = 13 and 8, respectively; Figure 5B). Pooled data are presented in Figure 5C, which shows that the increase in twitch peak shortening by ISO was significant ($p < 0.01$), and did not depend on the protocol used for cell stimulation ($p = 0.808$ for ISO × rhythm interaction).

As it can be seen in Figure 5B, the enhancement of twitch amplitude by ISO was proportionally greater for those contractions with lower amplitude (i.e., following shorter stimulus intervals). The double pulse

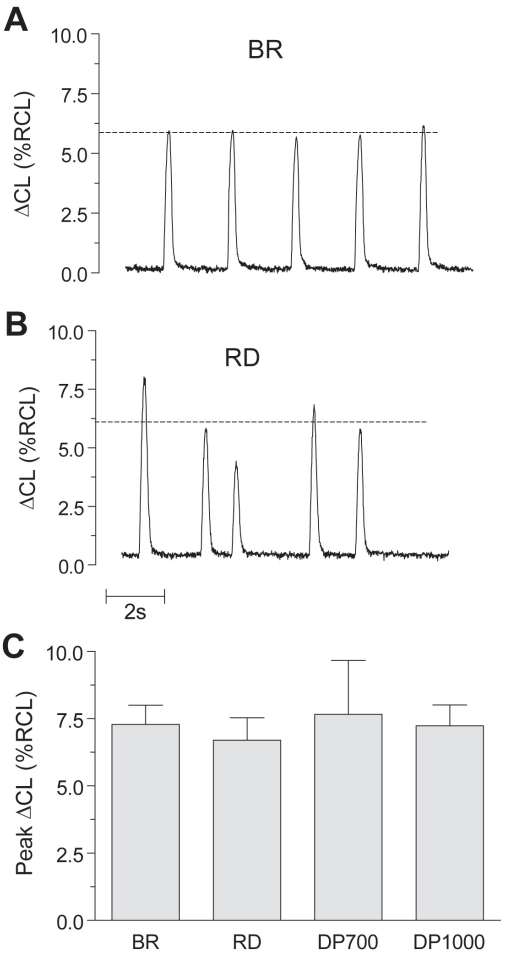

Figure 4. Stimulatory rhythm and contraction amplitude. Cell shortening (ΔCL) in rat ventricular myocytes, as percentage of the resting cell length (RCL), during stimulation with basic (BR, in A) and pseudo-random (RD, in B) rhythms. Dashed lines represent the average twitch amplitude. In C, mean (bars) and SEM (vertical lines) of average peak twitch shortening for all the four stimulatory patterns shown in Figure 2, of which the average rate was 0.5 Hz. Twitch amplitude was averaged in 10 successive contractions for each cell.

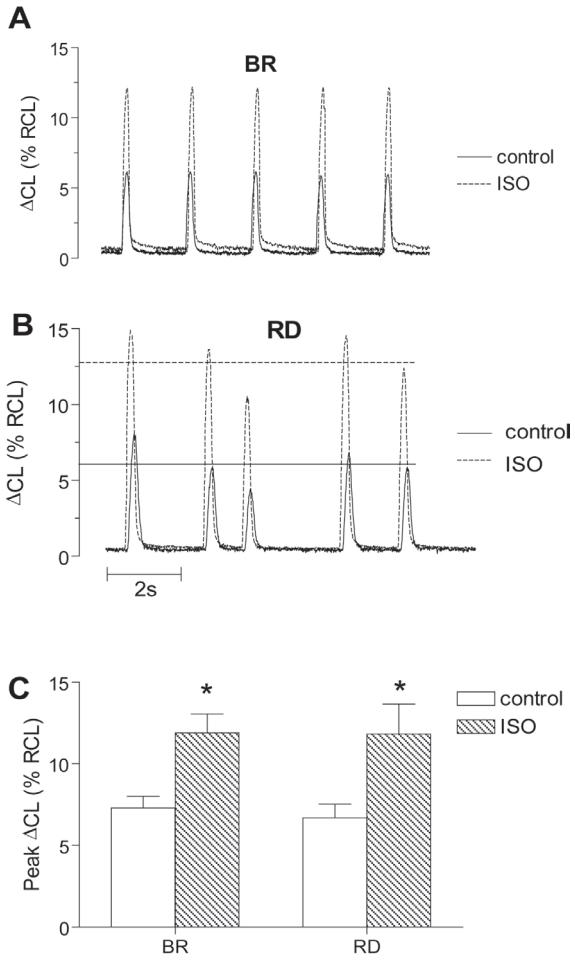

Figure 5. Effect of β-adrenergic stimulation on contraction at different stimulatory rhythms. Cell shortening (ΔCL) in ventricular myocytes, as percentage of the resting cell length (RCL), during stimulation with basic (BR, in A) and pseudo-random (RD, in B) rhythms in the absence (control, solid lines) and presence of 10 nM isoproterenol (ISO, dashed lines). The lower and upper horizontal lines in B represent the average twitch amplitude in the absence and presence of ISO, respectively. C) Means and SEM of average ΔCL at both stimulatory patterns, in the absence and presence of ISO. *, $p < 0.05$, Bonferroni test.

protocols (DP1000 and DP700) were used to study how ISO affects the amplitude ratio of two consecutive contractions separated by different time intervals. In these protocols, we will call TW1 and TW2 the twitches evoked after the longer and the shorter intervals, respectively. Figure 6 shows that, in the absence of ISO, twitch amplitude was markedly dependent on the preceding interval (8.9 ± 0.8 and 5.5 ± 0.7% of RCL for TW1 and TW2, respectively for DP1000, n = 13; 9.8 ± 2.2; and 5.5 ± 1.8% of RCL for TW1 and TW2, respectively for DP700, n = 4; p = 0.005; solid lines in Figures 6A and 6B). At steady-state, the average TW2/TW1 ratio was less than 1 and similar between DP protocols (0.59 ± 0.03 and 0.54 ± 0.06 for DP1000 and DP700, respectively, p = 0.509; control in Figure 6C). The average twitch amplitude was enhanced by ISO (from 7.3 ± 0.9 to 12.4 ± 1.3 for DP1000; from 7.7 ± 2.0 to 14.2 ± 1.4 for DP700; p = 0.001), although the potentiation was clearly greater for TW2 than for TW1 (dashed lines in Figures 6A and 6B). As a result, ISO

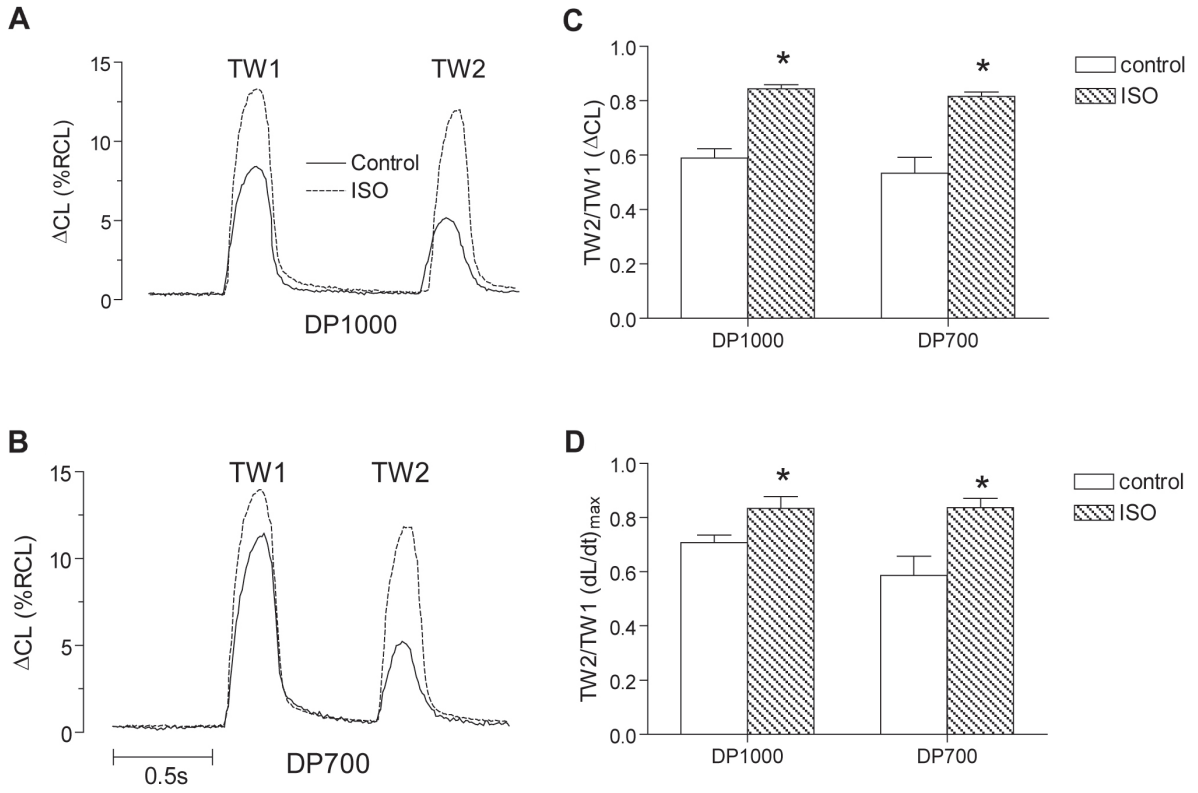

Figure 6. Interval-dependent effect of β-adrenergic stimulation on contraction. Cell shortening (ΔCL) in ventricular myocytes, as percentage of the resting cell length (RCL) during the application of the double pulse protocols DP1000 (A) and DP700 (B) in the absence (control, solid lines) and presence of 10 nM isoproterenol (ISO, dashed lines), where TW1 and TW2 are the contractions evoked after the longer and the shorter intervals, respectively. The mean ratios of TW2 and TW1 peak amplitude and maximal time-derivative of cell shortening ($(dL/dt)_{max}$) are shown in C and D, respectively. *, $p < 0.05$, Bonferroni test.

increased significantly the TW2/TW1 ratio (to 0.84 ± 0.01 and 0.82 ± 0.02, respectively; $p < 0.001$; Figure 6C), tending to equalize TW1 and TW2 amplitude. The effect of ISO on the maximum time-derivative of cell shortening ($(dL/dt)_{max}$) paralleled that on twitch amplitude (Figure 6D).

During β-adrenoceptor stimulation, cell relaxation was significantly faster ($t_{1/2\text{-rel}} = 85 \pm 7$ ms) compared to control ($t_{1/2\text{-rel}} = 121 \pm 12$ ms; $p < 0.001$; $n = 11$; Figure 7A) in cells stimulated with DP700. Accordingly, $(dL/dt)_{min}$ was significantly increased by the agonist ($p < 0.001$, Figure 7B). Similar effects were observed when cells were paced with the DP1000 protocol (not shown).

Discussion

The device developed in the present work (CCIP) can magnify the cell image by approximately 1,700 times. This is a reasonable amplification to work with cardiac myocytes, considering that the typical cell length ranges from 90 to 120 µm. Thus, a cell 100 µm long would be displayed on the video monitor as a 170 mm long image. The fact that parallax or optical distortions was not detected during bench tests makes the CCIP useful and reliable for measurement of cell movement, such as used in the experiments of the present application. In addition, the CCIP is simple to built and use, reasonably inexpensive (~US$1,000.00) and very compact, allowing the setup to be assembled in restricted laboratory space.

One of our main findings in this work was that, provided that the average stimulatory frequency is maintained constant, stimulus time-distribution does not affect the average peak contraction developed by ventricular myocytes. We have previously described similar results in isolated left atria paced at different rhythms and rates, namely, that the average rate, rather than the rhythm, is the main determinant of the interval-dependence of inotropic function (Bassani *et*

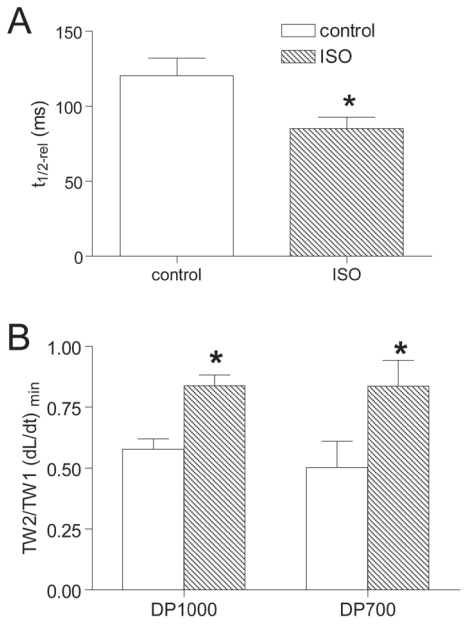

Figure 7. Effect of β-adrenergic stimulation on twitch relaxation. A) mean half-time for relaxation ($t_{1/2\text{-rel}}$) of TW1 (DP1000 protocol) in the absence (control) and presence of 10 nM isoproterenol (ISO). B) TW2/TW1 ratio of the maximal time-derivative of cell relaxation ($(dL/dt)_{min}$) in the presence and absence of ISO. *, $p < 0.05$, Student t test (A) and Bonferroni test (B).

al., 1990). The slow timecourse of Ca^{2+} transients and contractions at the temperature at which the present experiments were carried out (23 ºC, Puglisi *et al*., 1996) precludes the use of very short stimulation intervals. In spite of this limitation, it is remarkable that the same behavior observed in a multicellular atrial preparation can be reproduced in isolated ventricular cells. This indicates that the phenomenon is independent of the region of heart and, most importantly, that the frequency control of the developed force seems to be an intrinsic property of the cardiac myocyte. While heart rate variability *in vivo* is a vastly investigated topic, the rate × rhythm influence on cardiac inotropism has not received as much attention. Although the positive inotropic effect of electric stimulation with double pulse stimulation patterns has been reported (Wiggins *et al*., 1975), these patterns consist in the insertion of an extra stimulus for each regular pulse, so that the stimulation rate is actually doubled. Our observations (Bassani *et al*., 1990; present results) indicate that the positive inotropic effect of such patterns is likely to stem from the frequency change, rather than the alteration of rhythm.

As shown in Figures 4B and 6A-B, abbreviation of the interval between contractions produced weaker contractions than those after longer intervals. This result is consistent to other observations reported in the literature (Bers, 2001). After a given contraction, some time is required to elapse before another contraction with comparable amplitude can be evoked. This seems to depend not only on the time-dependent electric restitution (i.e., recovery of the action potential duration, which is one of the determinants of Ca^{2+} influx, Boyett & Jewell, 1978; Bassani *et al*., 2004), but also on the recovery of the sensitivity of the sarcoplasmic reticulum (SR) Ca^{2+} channels to activation by Ca^{2+}(Fabiato, 1985; Bers *et al*., 1993; Györke & Fill, 1993; Satoh *et al*., 1997). These factors are of paramount importance for the excitation-contraction coupling, because most of the Ca^{2+} that activates the myofilaments comes from the SR and is released in response to Ca^{2+} influx through voltage-dependent channels (Ca^{2+} current, I_{Ca}) during the action potential (Bers, 2001).

In mammalian myocardium, instantaneous shortening of the interval between contractions may elicit complex responses. In addition to changes in action potential duration due to time dependence of the biophysical properties of voltage-dependent ion channels (Boyett & Jewell, 1978; Bassani *et al*., 2004), less time for the SR Ca^{2+} release channels to recover from the inactivated/adapted state will result in smaller release of Ca^{2+} to the cytosol, and consequently reduced amplitude of the extrasystolic contraction. This seems to be the major factor limiting contraction amplitude, as the recovery from inactivation of sarcolemmal L-type Ca^{2+} channels and the recovery of the SR Ca^{2+} content are comparatively much faster (Bers, 2001). On the other hand, as I_{Ca} recovers more rapidly than SR Ca^{2+} release, Ca^{2+} influx during the extrasystolic contraction might be greater due to attenuation of Ca^{2+}-dependent inactivation of sarcolemmal Ca^{2+} channel by the Ca^{2+} released from the SR (Puglisi *et al*., 1999). This would result in uptake of the extra Ca^{2+} by the SR, so that more Ca^{2+} would be available for release at the next contraction. This seems to be the basis of the post-extrasystolic potentiation phenomenon (Bers, 2001).

The positive relationship between interval and contraction amplitude also exists for unphysiologically long intervals (many seconds), manifested as the post-rest potentiation phenomenon observed in the rat and ferret myocardium, and attributed to a slow phase of recovery of the SR channels (Bers *et al*., 1993; Bassani & Bers, 1994; Ferraz *et al*., 2001). In other species (e.g., rabbit) in which sarcolemmal Na^+/Ca^{2+} exchanger is

faster than in rodents and contributes to SR Ca depletion during rest, post-rest decay, rather than potentiation of twitch amplitude, and SR Ca loss are observed. However, inhibition of the exchanger during rest results in time-dependent facilitation of SR Ca^{2+} release and twitch potentiation, without apparent change in SR Ca load, as seen in rodent hearts (Bassani & Bers, 1994; Satoh *et al*., 1997). This indicates that the positive relationship between intervals and twitch amplitude is a basic phenomenon in mammalian myocardium, and that not all SR Ca^{2+} release channels are available at physiological heart rates. It is expected that the greater the number of silent channels, the lower will be the fraction of the SR Ca^{2+} content released (FR, Bassani *et al*., 1995a). Conversely, if time-dependent recovery of the channels proceeds to a greater extent, FR will be larger. Thus, if variable intervals occur in such a fashion that average frequency is nearly constant, the lower FR after shorter intervals would be compensated for greater release after longer intervals, so that, over time, the average FR, an thus contraction amplitude, would be approximately constant. On the other hand, if the average frequency is changed, other factors, such as alteration in the action potential waveform, intracellular Na^+ concentration (and thus Na^+/Ca^{2+} function), SR Ca^{2+} content, and modulation of I_{Ca}, as well as of SR Ca^{2+} release and uptake by Ca^{2+}-calmodulin-dependent kinase II (Boyett *et al*., 1987; Xiao *et al*., 1994; Bassani *et al*., 1995b, 2004; Li *et al*., 1997; Maier *et al*., 2000; Fauconnier *et al*., 2003) might contribute to cumulative modification of FR in a monotonic fashion, leading to changes in average contraction amplitude.

Another important finding in this work was that ISO tended to reduce the influence of the interval on twitch amplitude, increasing more the amplitude of the smaller contractions after short intervals (Figures 5 and 6). The positive inotropic effect of β-adrenergic stimulation may be attributed to increase in Ca^{2+} influx and SR Ca^{2+} uptake (which can be inferred from acceleration of relaxation, Figure 7), which augments the SR Ca^{2+} store, an important determinant of FR (Bassani *et al*., 1995a). These effects are mediated by substrate phosphorylation by the protein kinase A (PKA), the intracellular effector of the β-adrenergic cascade (Bers, 2001). At regular rhythm, ISO increases FR by enhancing both I_{Ca} and SR Ca^{2+} content, but not by modulating the release process itself (Ginsburg & Bers, 2004). A likely possibility to explain the greater effect of ISO on twitches following shorter interval would be acceleration of recovery from adaptation of the SR release channels by PKA phosphorylation (Valdivia *et al*., 1995). Such recovery seems to be the limiting factor for time dependence of SR Ca^{2+} release, and its acceleration would be expected to increase FR more after short than long intervals.

Heart rate *in vivo* undergoes cyclic oscillations attributed to autonomic modulation and changes in intrathoracic pressure associated with pulmonary ventilation (Aubert & Ramaekers, 1999; Stein & Kleiger, 1999; Yasuma & Hayano, 2004; Lewis, 2005). In the latter cases, it is believed that heart rate variability in synchrony with respiration has an important role in allowing an efficient match of lung ventilation and perfusion (Yasuma & Hayano, 2004). Heart rate variability is decreased in ischemic heart disease, congestive heart failure and diabetes, and it has been proposed as an important indicator for prognosis in patients with cardiac diseases (Aubert & Ramaekers, 1999; Stein & Kleiger, 1999). Our present results are suggestive that, while beat-to-beat changes in ventricular developed force are likely to occur *in vivo*, the average contractile performance would be maintained if the average rate remains approximately constant. Thus, cardiac contractile activity *in vivo* would be ever changing and far from steady state, however dynamically stable for a given average heart rate.

The effect of sympathetic stimulation of inotropism was mimicked by exposure to ISO at a concentration close to that required for half-maximal stimulation. It is well known that the activation of β-adrenoceptors by sympathetic mediators increases not only myocardial contraction force, but also conduction velocity and heart rate (Bers, 2001). One would expect that the latter effect would require changes in electric and mechanical function, so as to match the decrease in spontaneous cycle length. Acceleration of the decline of the Ca^{2+} transient and mechanical relaxation (Bassani *et al*., 1995b; present results), as well as shortening of action potential and electric refractoriness (Bers, 2001), contribute to adaptation of contractile activity to high heart rates. Our present observation that ISO exerts a comparatively larger inotropic effect on contractions preceded by short intervals (possibly by accelerating recovery of SR Ca^{2+} release) deserves further investigation, as it may represent a novel mechanism by which β-adrenergic stimulation enables the heart to enhance the contractile performance in spite of abbreviation of the diastolic interval.

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References

Aubert, A.E., Ramaekers, D. (1999), "Neurocardiology: the benefits of irregularity. The basics of methodology, physiology and current clinical applications", *Acta Cardiologica*, v. 54, p. 107-120.

Bassani, J.W.M., Costa, E.T., Rocha-e-Silva, M. (1990), "A portable microprocessor-based triggering device for cardiac muscle electrical stimulation", *Brazilian Journal of Medical and Biological Research*, v. 23, p. 87-91.

Bassani, J.W.M., Yuan, W., Bers, D.M. (1995a), "Fractional SR Ca release is regulated by trigger Ca and SR Ca content in cardiac myocytes", *American Journal of Physiology*, v. 268, p. C1313-C1319.

Bassani, R.A., Bers, D.M. (1994), "Na-Ca exchange is required for rest-decay but not for rest potentiation of twitches in rabbit and rat ventricular myocytes", *Journal of Molecular and Cell Cardiology*, v. 26, p. 1335-1347.

Bassani, R.A., Mattiazzi, A., Bers, D.M. (1995b), "CAMKII is responsible for activity-dependent acceleration of relaxation in rat ventricular myocytes", *American Journal of Physiology*, v. 268, p. H703-H712.

Bassani, R.A., Bassani, J.W.M. (2002), "Contribution of Ca^{2+} transporters to relaxation in intact ventricular myocytes from developing rats", *American Journal of Physiology*, v. 282, p. H2406-H2413.

Bassani, R.A., Altamirano, J., Puglisi, J.L., Bers, D.M. (2004), "Action potential duration determines sarcoplasmic reticulum Ca^{2+} reloading in mammalian ventricular myocytes", *Journal of Physiology*, v. 559, p. 591-607.

Bers, D.M. (2001), *Excitation-Contraction Coupling and Cardiac Contractile Force*, 2nd ed. Dordretch: Kluwer Academic Publishers.

Bers, D.M., Bassani, R.A., Bassani, J.W.M., Baudet, S., Hryshko, L.V. (1993), "Paradoxical twitch potentiation after rest in cardiac muscle: increased fractional release of SR calcium", *Journal of Molecular and Cellular Cardiology*, v. 25, p. 1047-1057.

Bowditch, H.P. (1871), "Über die Eigenthümlichkeiten der Reizbarkeit, welche die Muskelfasern des Herzens zeigen", *Mathematisch-Physische Klasse*, v. 23, p. 652-689.

Boyett, M.R., Jewell, B.R. (1978), "A study of the factors responsible for rate-dependent shortening of the action potential in mammalian ventricular muscle", *Journal of Physiology*, v. 285, p. 359-380.

Boyett, M.R., Hart, G., Levi, A.J., Roberts, A. (1987), "Effects of repetitive activity on developed force and intracellular sodium in isolated sheep and dog Purkinje fibres", *Journal of Physiology*, v. 388, p. 295-322.

Fabiato, A. (1985), "Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell", *Journal of General Physiology*, v. 85, p. 247-289.

Fauconnier, J., Bedut, S., Le Guennec, J.Y., Babuty, D., Richard, S. (2003), "Ca^{2+} current-mediated regulation of action potential by pacing rate in rat ventricular myocytes", *Cardiovascular Research*, v. 57, p. 670-680.

Ferraz, S.A., Bassani, J.W.M., Bassani, R.A. (2001), "Rest-dependence of twitch amplitude and sarcoplasmic reticulum calcium content in the developing rat myocardium", *Journal of Molecular and Cellular Cardiology*, v.33, p. 711-722.

Ginsburg, K.S., Bers, D.M. (2004), "Modulation of excitation-contraction coupling by isoproterenol in cardiomyocytes with controlled SR Ca^{2+} load and Ca^{2+} current trigger", *Journal of Physiology*, v. 556, p. 463-480.

Györke, S., Fill, M. (1993), "Ryanodine receptor adaptation: control mechanism of Ca^{2+}-induced Ca^{2+} release in heart", *Science*, v. 260, p. 807-809.

Hajdu, S. (1953), "Mechanism of staircase and contracture in ventricular muscle", *American Journal of Physiology*, v. 174, p. 371-380.

Hoffman, B.F., Kelly, J.J. Jr. (1959), "Effects of rate and rhythm on contraction of rat papillary muscle", *American Journal of Physiology*, v. 197, p. 1199-1204.

Koch-Weser, J., Blinks, J.R. (1963), "The influence of the interval between beats on myocardial contractility", *Pharmacological Reviews*, v. 15, p. 601-652.

Lakatta, E.G. (2004), "Beyond Bowditch: the convergence of cardiac chronotropy and inotropy", *Cell Calcium*, v. 35, p. 629-642.

Lewis, M.J. (2005), "Heart rate variability analysis: a tool to assess cardiac autonomic function", *Computers and Informatics in Nursing*, v. 23, p. 335-341.

Li, L., Satoh, H., Ginsburg, K.S., Bers, D.M. (1997), "The effect of Ca^{2+}-calmodulin-dependent protein kinase II on cardiac excitation-contraction coupling in ferret ventricular myocytes", *Journal of Physiology*, v. 501, p. 17-31.

Maier, L.S., Bers, D.M., Pieske, B. (2000), "Differences in Ca^{2+}-handling and sarcoplasmic reticulum Ca^{2+}-content in isolated rat and rabbit myocardium", *Journal of Molecular and Cellular Cardiology*, v. 32, p. 2249-2258.

Puglisi, J.L., Bassani, R.A., Bassani, J.W.M., Amin, J.N., Bers, D.M. (1996), "Temperature and relative contributions of Ca transport systems in cardiac myocyte relaxation. *American Journal of Physiology*, v. 270, p. H1772-H1778.

Puglisi, J.L., Yuan, W., Bassani, J.W.M., Bers, D.M. (1999), "Ca^{2+} influx through Ca^{2+} channels in rabbit ventricular myocytes during action potential clamp: influence of temperature", *Circulation Research*, v. 85, p. e7-e16.

Satoh, H., Blatter, L.A., Bers, D.M. (1997), "Effect of $[Ca^{2+}]i$, SR Ca^{2+} load, and rest on Ca^{2+} spark frequency in ventricular myocytes", *American Journal of Physiology*, v. 272, p. H657-H668.

Steadman, B.W., Moore, K.B., Spitzer, K.W., Bridge, J.H. (1988), "A video system for measuring motion in contracting heart cells", *IEEE Transactions in Biomedical Engineering*, v. 35, p. 264-272.

Stein, P.K., Kleiger, R.E. (1999), "Insights from the study of heart rate variability", *Annual Review of Medicine*, v. 50, p. 249-261.

Valdivia, H.H., Kaplan, J.H., Ellis-Davies, C.G.R., Lederer, W.J. (1995), "Rapid adaptation of cardiac ryanodine receptors: modulation by Mg^{2+} and phosphorylation", *Science*, v. 267, p. 1997-2000.

Wiggins, J.R., Leary, J.M., Cranefield, P.F. (1975), "The effects of verapamil and paired-pulse stimulation on mammalian ventricle", *European Journal of Cardiology*, v. 3, p. 181-185.

Xiao, R.P., Cheng, H., Lederer, W.J., Suzuki, T., Lakatta, E.G. (1994), "Dual regulation of Ca^{2+}/calmodulin-dependent kinase II activity by membrane voltage and by calcium influx", *Proceedings from the National Academy of Sciences*, USA, v. 91, p. 9659-9663.

Yasuma, F., Hayano, J. (2004), "Respiratory sinus arrhythmia: why does the heartbeat synchronize with respiratory rhythm?", *Chest*, v. 125, p. 683-690.