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SIGNALING AND CELL PHYSIOLOGY

The reduced myofilament responsiveness to calcium contributes to the negative force-frequency relationship in rat cardiomyocytes: role of reactive oxygen species and p-38 map kinase

María Sofía Espejo[1] · Ignacio Aiello[1] · Marisa Sepúlveda[1] · Martín G. Vila Petroff[1] · Ernesto A. Aiello[1] · Verónica C. De Giusti[1]

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Abstract The force-frequency relationship (FFR) is an important intrinsic regulatory mechanism of cardiac contractility. However, a decrease (negative FFR) or no effect (flat FFR) on contractile force in response to an elevation of heart rate is present in the normal rat or in human heart failure. Reactive oxygen species (ROS) can act as intracellular signaling molecules activating diverse kinases as calcium-calmodulin-dependent protein kinase II (CaMKII) and p-38 MAP kinase (p-38K). Our aim was to elucidate the intracellular molecules implicated in the FFR of isolated rat ventricular myocytes. The myocytes were field-stimulated via two-platinum electrodes. Sarcomere length was recorded with a video camera. Ca^{2+} transients and intracellular pH_i were recorded by epifluorescence. Increasing frequency from 0.5 to 3 Hz decreased cell shortening without changes in pH_i. This negative FFR was changed to positive FFR when the myocytes were pre-incubated with the ROS scavenger MPG, the NADPH oxidase blocker apocynin, or by inhibiting mitochondrial ROS production with 5-HD. Similar results were obtained when the cells were pre-incubated with the CaMKII blocker, KN-93, or the p-38K inhibitor, SB-202190. Consistently, the levels of phosphorylation of p-38K and the oxidation of CaMKII were significantly higher at 2 Hz than at 0.5 Hz. Despite the presence of positive inotropic effect during stimulation frequency enhancement, Ca^{2+} transient amplitudes were reduced in MPG- and SB-202190-treated myocytes. In conclusion, our results indicate that the activation of the intracellular pathway involving ROS-CaMKII-p-38K contributes to the negative FFR of rat cardiomyocytes, likely by desensitizing the response of contractile myofilaments to Ca^{2+}.

Keywords Rat ventricular myocytes · Reactive oxygen species · p38 MAP kinase · Negative staircase · Contractility

Introduction

The force-frequency relationship (FFR) is an important intrinsic regulatory mechanism of cardiac contractility, which represents a key adaptation for increasing cardiac output in response to enhanced systemic demands. The FFR in most mammalian species is positive, which means that an increase in the frequency of stimulation induces an increase in the force of contraction. However, in some species, like the rat [33], or under pathological conditions, as human heart failure [35, 45], the existence of a negative or flattened FFR has been demonstrated, which means that an increase in the frequency of stimulation generates a decrease or no response in the force of contraction, respectively [20].

For many years, researchers have been trying to explain the causes of the negative FFR in the rat. It has been demonstrated a clear negative FFR in rat from 0.25 to 3 Hz, which is not paralleled by a reduction in sarcoplasmic reticulum (SR) Ca^{2+} load. In this context, it has been shown that rat myocytes exhibit higher intracellular Na^+ (Na^+_i) concentration compared to other species. High Na^+_i levels would favor Ca^{2+}

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✉ Ernesto A. Aiello
aaiello@ciclaplata.org.ar

✉ Verónica C. De Giusti
vdegiusti@med.unlp.edu.ar

[1] Centro de Investigaciones Cardiovasculares "Dr. Horacio E. Cingolani", Facultad de Ciencias Médicas, Universidad Nacional de La Plata-CONICET, Calle 60 y 120, 1900 La Plata, Argentina

influx or decrease Ca^{2+} efflux through the Na/Ca exchanger (NCX), increasing intracellular calcium (Ca^{2+}_i) concentration and loading the SR close to its maximal level. The high Ca^{2+}_i helps to maintain the contractility at low stimulation frequencies, but would also be detrimental at higher heart rates, when the SR will not be able to take up more Ca^{2+} [33]. Also, it has been demonstrated that the intracellular pH (pH_i) could be altered by an increase in the frequency of stimulation [10], and this might influence the contractile response. In the present work, we measure simultaneously pH_i and sarcomere shortening in response to an increase in the frequency of stimulation from 0.5 to 2 Hz.

In the last few years, evidence has emerged supporting the role of the reactive oxygen species (ROS) as second messengers. The major sources of ROS in cardiomyocytes are the NADPH oxidase (NOX) [4] and the mitochondrial respiratory chain [9, 17, 19, 55]. A crosstalk between NOX and mitochondria has been demonstrated, which might maximize the production of ROS [18]. In 2000, Zorov et al. published the first study describing the phenomenon called "ROS-induced ROS-release" by which a small amount of ROS triggers greater ROS production from the mitochondria [59]. The main link between both sources of ROS (NOX and mitochondria) seems to be the mitochondrial ATP-dependent potassium channel ($mitoK_{ATP}$) [26, 40, 41, 43, 56]. It was described that the opening of these channels is crucial to stimulate ROS production by the respiratory chain. Increasing evidence demonstrated that ROS mediate the inotropic effect of different extracellular hormones, as angiotensin II (Ang II) [12] or endothelin-1 (ET-1) [13]. Moreover, it has been shown that the increase in the pacing frequency stimulates ROS production [24, 50]. In addition, ROS activate diverse kinases as p-38 MAP kinase (p-38K) and calcium-calmodulin-dependent protein kinase II (CaMKII) [46]. p-38K was demonstrated to mediate the negative inotropic effect of Ang II [44], possibly due to a reduction in myofilament responsiveness to Ca^{2+} [44]. Lastly, CaMKII was shown to stimulate p-38K pathway [46]. As the involvement of ROS and these kinases during the force-frequency relationship in the rat myocytes has not been fully studied, the aim of this investigation was to elucidate if the pathway ROS-CaMKII-p-38K is involved in the generation of the negative FFR and if so, to determine whether alterations in Ca^{2+} transient and/or myofilament responsiveness mediate such effect.

Materials and methods

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of La Plata University. For all the experiments, male Wistar rats of 4 months of age were used.

Myocyte isolation

Rat myocytes were isolated according to the technique previously described [1, 36] with some modifications. Briefly, the hearts were attached via the aorta to a cannula, excised, and mounted in a Langendorff apparatus. They were then retrograde perfused at 37 °C at a constant perfusion pressure of 70–80 mmHg with Krebs-Henseleit (K-H) solution of the following composition (in mM): 146.2 NaCl, 4.7 KCl, 1 $CaCl_2$, 10 HEPES, 0.35 NaH_2PO_4, 1 $MgSO_4$, and 10 glucose (pH adjusted to 7.4 with NaOH). The solution was continuously bubbled with 100% O_2. After a stabilization period of 4 min, the perfusion was switched to a nominally Ca^{2+}-free K-H for 6 min. Hearts were then recirculated with collagenase (118 units/ml), 0.1 mg/ml protease, and 1% BSA in K-H containing 50 μM $CaCl_2$. Perfusion continued until hearts became flaccid (15–25 min). Hearts were then removed from the perfusion apparatus by cutting at the atrioventricular junction. The desegregated myocytes were separated from the undigested tissue and rinsed several times with a K-H solution containing 1% BSA and 500 μM $CaCl_2$. After each wash, myocytes were left for sedimentation for 10 min. Myocytes were kept in K-H solution at room temperature (20–22 °C) until use, when they were changed to a bicarbonate solution bubbling with 5% CO_2 and 95% O_2. Only rod-shaped myocytes with clear and distinct striations and an obvious marked shortening and relaxation on stimulation were used. Experiments were performed at room temperature.

Sarcomere shortening

In order to measure sarcomere length (SL), myocytes were placed in a perfusion chamber on the stage of an inverted microscope (Nikon TE 2000-U) and continuously superfused with a solution containing (mM) 5 KCl, 118 NaCl, 1.2 $MgSO_4$, 0.8 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 20 $NaHCO_3$, and pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2 (bicarbonate solution). The myocytes were stimulated via two-platinum electrodes on either side of the bath at 0.5, 1, 2, and 3 Hz. The SL was recorded by specific software (ION WIZARD analysis software). The myocytes were observed using a video camera connected to the microscope. The SL was measured in a determined region of the myocyte. The software estimated the most frequent SL in that region using fast Fourier transform analysis (Ion Optix, Milton, MA). The experiments were performed at room temperature.

Calcium transient

Sarcomere shortening and free Fura-2 and Fura-2 bound calcium fluorescence were measured simultaneously. Myocytes were loaded with 10 μM Fura-2 AM during 12 min. Fura-2 fluorescence was measured on an inverted microscope

adapted for epifluorescence by an IonOptix hardware. Cells were continuously superfused with bicarbonate-buffered solution containing (mM) 5 KCl, 118 NaCl, 1.2 $MgSO_4$, 0.8 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 20 $NaHCO_3$, and pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2 at a constant flow of 1 ml/min and field stimulated at 0.5, 1, 2, and 3 Hz via two-platinum electrodes on either side of the bath. The ratio of the free Fura-2 and Fura-2 bound calcium fluorescence obtained after exciting the dye at 340 and 380 nm was taken as an index of $Ca^{2+}{}_i$.

Free Fura-2 and Fura-2 bound calcium ratio-SL shortening phase-plane diagrams (loops)

As previously reported [53], the $[Ca^{2+}]_i$-cell length trajectory during the relaxation phase of the twitch contraction in single cardiac myocytes appears to define a quasi-equilibrium of cytosolic $[Ca^{2+}]$, myofilament Ca^{2+} binding, myofilament force, and thus cell length. The relative position of the trajectory reflects the relative myofilament Ca^{2+} response; a shift in the position of the trajectory reflects a shift in the myofilament Ca^{2+} response. SL shortening relates to $[Ca^{2+}]_i$ in a hysteresis loop fashion. Under our experimental conditions, this loop is formed by plotting SL shortening vs free Fura-2 and Fura-2 bound calcium ratio (F2r). We studied the relaxation phase of this loop fitting the Hill equation with offset as the following:

$$\text{SL shortening} = \text{SL0}\ [(\text{F2r})^n/(k^n + (\text{F2r})^n] + \text{offset}$$

whereas SL0 is the maximum active SL shortening, k may be the $[Ca^{2+}]_i$ (F2r) where 50% of maximum SL shortening is reached, and n may be a measure of calcium-dependent cooperativity. We then differentiated the k point to obtain the *loop relaxation slope* which was used as an index of myofilament calcium sensitivity.

pH_i measurements during the increase in the frequency of stimulation

pH_i was measured in single myocytes with an epifluorescence system (Ion Optix, Milton, MA). Myocytes were incubated at room temperature for 10 min with 10 μM BCECF-AM followed by 30 min washout. Dye-loaded cells were placed in a chamber on the stage of an inverted microscope (Nikon.TE 2000-U) and continuously superfused with a solution containing (mM) 5 KCl, 118 NaCl, 1.2 $MgSO_4$, 0.8 Cl_2Mg, 1.35 Cl_2Ca, 10 glucose, 20 $NaHCO_3$, and pH 7.4 after continuous bubbling with 5% CO_2 and 95% O_2. The myocytes were stimulated via two-platinum electrodes on either side of the bath from 0.5 to 2 Hz. Dual excitation (440 and 495 nm) was provided by a 75-W Xenon arc lamp and transmitted to the myocytes. Emitted fluorescence was collected with a photomultiplier tube equipped with a band-pass filter centered at 535 nm. The 495 to 440 nm fluorescence ratio was digitized at 10 kHz (ION WIZARD fluorescence analysis software). At the end of each experiment, the fluorescence ratio was converted to pH by in vivo calibrations using the high K^+-nigericin method [47].

Intracellular ROS in isolated myocytes

Intracellular ROS production was measured on a Zeiss 410 microscope using rat ventricular myocytes loaded with 5 μmol/l 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCF DA, Molecular Probes) for 30 min at 37 °C as previously described [50]. Expanded description can be found in the Electronic Supplementary Material.

Western blot

Preparation of hearts

Langendorff perfusion: Isolated rat hearts were perfused according to the Langendorff technique with Krebs-Henseleit (K-H) solution of the following composition (in mM): 146.2 NaCl, 4.7 KCl, 1 $CaCl_2$, 10 HEPES, 0.35 NaH_2PO_4, 1 $MgSO_4$, and 10 glucose (pH adjusted to 7.4 with NaOH). The solution was continuously bubbled with 100% O_2 and maintained at constant temperature (37 °C) and coronary flow (12–14 ml/min) as previously described (1). Experimental protocol: After stabilization, hearts were paced at 0.5 and 2 Hz for 1 min. For kinase inhibition, hearts were treated with inhibitor KN-93 2.5 μM during the stabilization period and for the entire pacing period. After pacing for 1 min at the different pacing frequencies either in the absence or presence of inhibitors, hearts were freeze-clamped and stored at −80 °C for biochemical assays.

Gel electrophoresis and immunoblot analysis

SDS-PAGE was performed in a criterion gel system (Bio-Rad). The resolving gels were on 10%. The gels were run at 200 V constant voltage for ~ 1 h in standard Laemmli running buffer (2). After the gels had finished running, they were immediately placed in transfer buffer to equilibrate. The gels were transferred onto 0.2 μm PVDF membrane (Inmobilon Millipore) at 60 V constant voltage for 1 h.

Blots were blocked overnight at 4 °C in 5% nonfat dry milk diluted in TBST (50 mM Tris-base, 200 mM NaCl, and 0.05% Tween-20, pH 7.5). Primary monoclonal antibody dilutions were used to detect oxidized CaMKII and phosphorylated p-38K in rat heart cell lysates. Specific antibodies against oxidized Met281/282 residue CaMKII (1:1000, Millipore), anti-CaMKII total (1: 1000, Abcam), Tyr189 residue p-38K (1:1000, Cell Signaling Technology, EE.UU), anti-p-38K total (1:1000, Cell Signaling Technology, EE.UU) were employed. Primary antibodies were incubated at 4 °C overnight. The blots were then washed and incubated in secondary antibody

(goat anti-rabbit and goat anti-mouse, Santa Cruz Biotechnology) for 2 h at room temperature. The secondary antibody was diluted 1:20,000 in the same dilution buffer as the primary. After being washed, the blots' immunoreactivity was visualized by a peroxidase-based chemiluminescence detection kit (Immobilon Western Millipore) using a Chemidoc Imaging System. The signal intensity of the bands was quantified using ImageJ (NIH).

Statistical analysis

Unpaired Student's *t* test or one-way ANOVA was used for statistical comparisons when appropriate. Data are expressed as means ± SEM. Differences were considered significant at $p \leq 0.05$. The *n* value was determined by the number of myocytes. The number of rats used to obtain these myocytes was also indicated for each treatment.

Drugs

For all the functional experiments, the drugs were added 10 min before the recordings. Figure S1 shows the basal effects of these drugs on percentage sarcomere shortening. The compounds employed did not significantly alter this parameter, with the exception of KN-93, which significantly decreased basal contractility by approximately 25%.

Results

Force-frequency relationship in isolated ventricular myocytes of rats

In order to study the effect of increasing the frequency of stimulation on contractility, myocytes were successively stimulated from 0.5 to 3 Hz and the sarcomere length shortening was recorded. As illustrated in Fig. 1, the increase in the frequency of stimulation induced a decrease in cell contraction, representing a negative force-frequency relationship (FFR). Versus 0.5 Hz, contractility at 2 Hz decreased approximately 18% (Fig. 1b). Figure S2 shows that diastolic sarcomere length slightly decreased at 2 Hz in comparison to 0.5 Hz, and this effect was observed under all experimental conditions studied (i.e., in the presence of the drugs used throughout the study). Since this negative FFR could be induced by a potential decrease in intracellular pH (pH_i) secondary to the increased metabolism, we loaded the myocytes with a fluorescent pH indicator (BCECF-AM, 10 μM) to measure pH_i, and sarcomere shortening was recorded simultaneously. While a clear negative inotropic effect was observed during the increase in stimulation frequency, no modification of the pH_i values was detected, suggesting that the negative FFR is independent of changes in pH_i (Fig. 2).

Role of reactive oxygen species in the development of the negative FFR

In cardiac myocytes, the principal sources of ROS are NOX and mitochondria. Moreover, the mitoK$_{ATP}$ is a major candidate involved in the stimulation of the mitochondrial ROS production. As ROS are the intracellular messengers of several physiological pathways, we investigated their participation in the negative FFR. Figure 3 shows that the negative response induced by the increase in the frequency of stimulation switched to a positive one after pre-incubation of myocytes with the ROS scavenger, N-(2-Mercapto-propionyl)-glycine (MPG; 2 mM), or by the NOX blocker, apocynin (Apo, 300 μM). Moreover, when myocytes were pre-incubated with the mitoK$_{ATP}$ blocker, 5-hydroxydecanoic acid (5-HD; 500 μM), the negative FFR was also replaced by a positive one, indicating that the ROS-induced ROS release mechanism is participating in the development of the negative FFR.

In order to confirm that the enhancement of stimulation frequency stimulates ROS production, direct measurements of ROS by DCF fluorescence were performed. Figure S3 shows that ROS production at 2 Hz increased 1.6-fold in comparison to that recorded at 0.5 Hz. These results are consistent with those obtained in previous studies [24, 50].

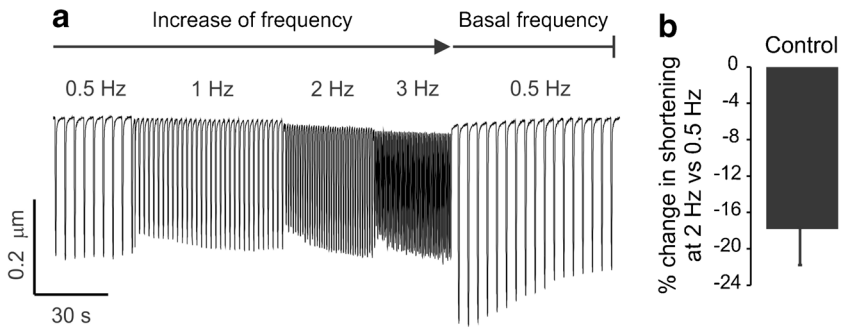

Fig. 1 **a** FFR in control conditions (HCO_3^--buffered solution). Representative traces of sarcomere length shortening during the increase of stimulation frequency (from 0.5 to 3 Hz) in control condition. **b** Average change in sarcomere length shortening, expressed as percentage of change in shortening at 2 vs 0.5 Hz (5 rats, *n* = 18)

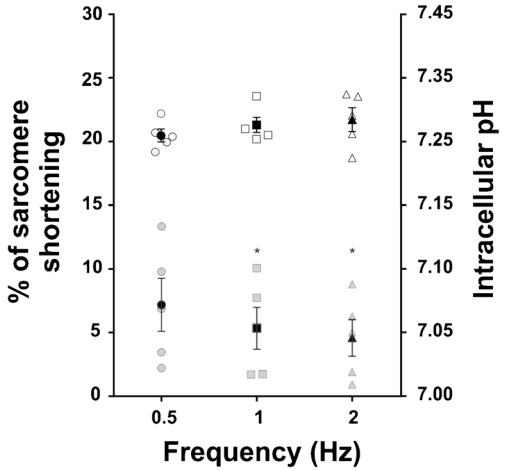

Fig. 2 Correlation between the increase in stimulation frequency and pH_i. Average data during the increase in stimulation frequency from 0.5 to 2 Hz. While the cells exhibited a clear negative FFR (*gray shapes*; 3 rats, $n = 5$), the pH_i did not change (*white shapes*; 3 rats, $n = 5$). Asterisk indicates $p < 0.05$ vs 0.5 Hz

Role of CaMKII and p-38K in the development of the negative FFR

It has been described that p-38K elicits a negative inotropic effect (NIE), which can occur without changes in calcium channel currents (L-type Ca^{2+}) or calcium transients, suggesting that p-38K may act by reducing myofilament calcium sensitivity [11, 28, 44]. In addition, p-38K is known to mediate the NIEs induced by angiotensin II (Ang II) [44]. Moreover, it has been demonstrated that CaMKII, which is known to be activated by ROS, can stimulate p-38K [46]. Thus, we next investigated if these kinases are involved in the negative FFR of the rat ventricular myocytes. When the cells were pre-incubated with the CaMKII blocker, KN-93 (2.5 μM, Fig. 4a), or with the p-38K blocker, SB-202190 (10 μM, Fig. 4b), the negative FFR was reverted to a positive one. In order to confirm the participation of these kinases during the change in frequencies, we studied the phosphorylation of p-38K and the oxidation of CaMKII at 0.5 and 2 Hz. Figure 5 shows that at 2 Hz, the levels of phosphorylation of p-38K and oxidation of CaMKII were significantly higher than at 0.5 Hz. Furthermore, the phosphorylation of p-38K was prevented with KN-93 (Fig. 5), suggesting that this kinase is activated downstream of CaMKII, as previously reported [46].

Changes in calcium transient during the increase in the frequency of stimulation

In order to stablish if the effect on the contractile response is due to changes in calcium handling, we loaded the myocytes with a fluorescent calcium indicator (Fura-2) and measured calcium transients during the increase in stimulation frequency. Figure 6 depicts simultaneous individual representative traces of sarcomere shortening and calcium transients in control condition and in the presence of MPG or SB-202190. In these loaded cells, the increase in stimulation frequency did not induce a change in contractility under control conditions

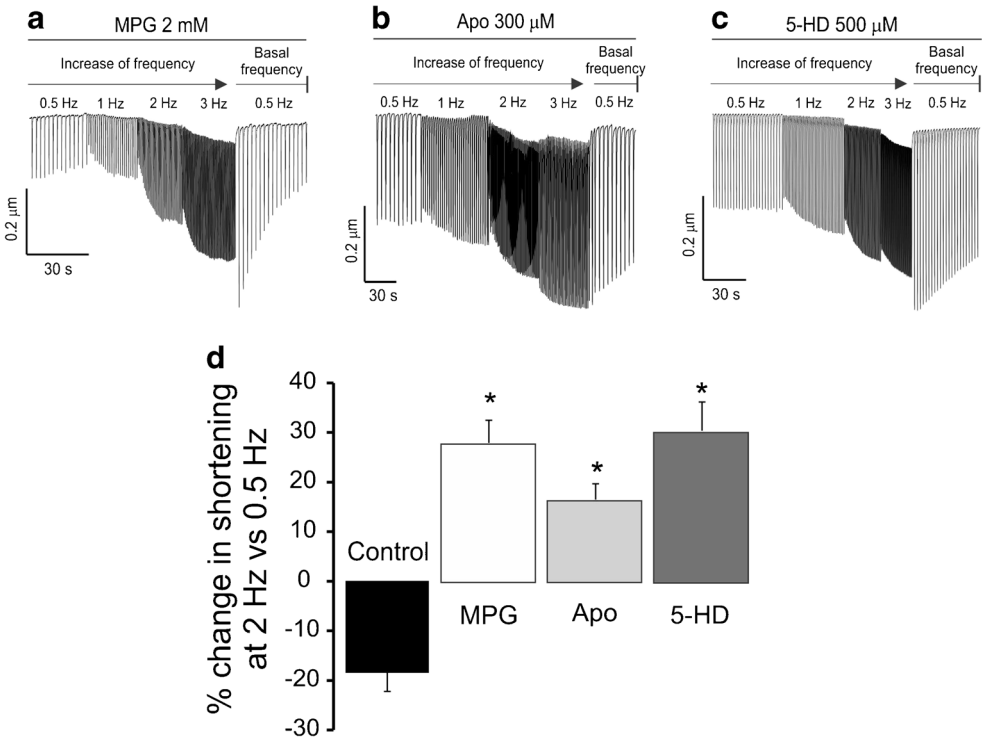

Fig. 3 Role of ROS during the FFR. Representative traces of cell shortening during the increase in stimulation frequency from 0.5 to 3 Hz in the presence of the ROS scavenger MPG (4 rats, $n = 11$; **a**), the NADPH oxidase blocker Apo (3 rats, $n = 10$; **b**), or the mitoK$_{ATP}$ blocker 5-HD (3 rats, $n = 6$; **c**). **d** shows average data expressed as percentage of change in shortening at 2 vs 0.5 Hz in the three previous conditions in comparison to control. Asterisk indicates $p < 0.05$ vs control

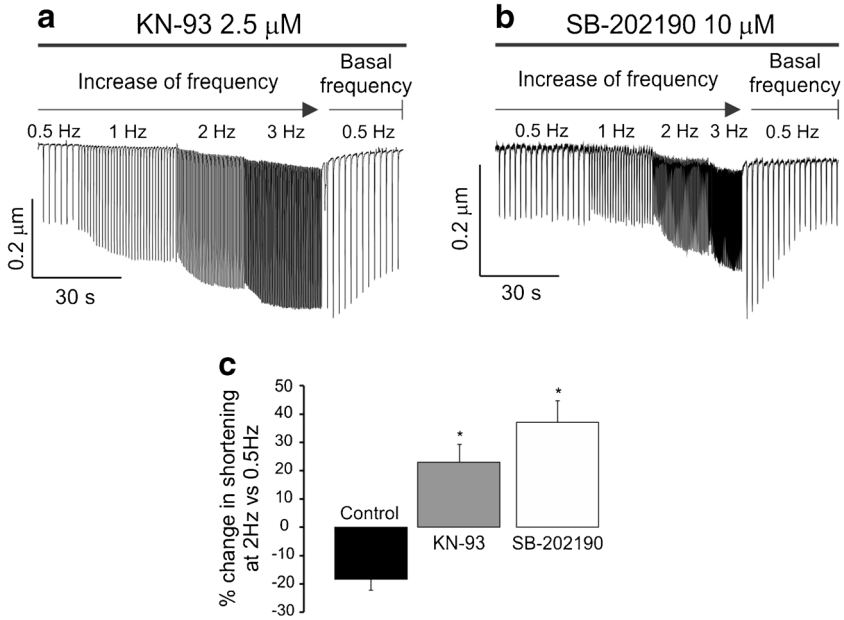

Fig. 4 Role of CaMKII and p-38K during the FFR. Representative traces of cell shortening during the increase in stimulation frequency from 0.5 to 3 Hz in the presence of the CaMKII blocker, KN-93 (3 rats, $n = 7$; **a**), or the p-38K blocker, SB-202190 (3 rats, $n = 7$; **b**). **c** shows average data expressed as percentage of change in cell shortening at 2 vs 0.5 Hz in both previous conditions in comparison to control. Asterisk indicates $p < 0.05$ vs control

(representing a flat FFR; Fig. 6a). On the other hand, similarly to the data obtained in the unloaded cells, a clear positive FFR was observed in the presence of the ROS scavenger and the p-38K blocker (Fig. 6b, c). However, the amplitudes of the Ca^{2+} transients recorded at 2 Hz were significantly lower than those registered at 0.5 Hz in all the experimental conditions studied (Fig. 6a–c). These results suggest that the ROS-induced p-38K stimulation might mediate desensitization in the response of contractile myofilaments to Ca^{2+}.

In order to confirm the involvement of ROS and p-38K in the desensitizing negative inotropic effect induced by the increase in stimulation frequency, the sarcomere length shortening/free Fura-2 and Fura-2 bound calcium ratio loops were performed in paired myocytes at 0.5 and 2 Hz. Figure 7 shows representative traces and average data obtained from the slope of the fitted relaxation phase of these loops as an index of myofilament Ca^{2+} sensitivity. Under control conditions, increasing frequency did not significantly alter the myofilament Ca^{2+} sensitivity, which in turn was clearly enhanced by the presence of SB202190 or MPG, reflected by the strong effect of these drugs on the loop relaxation slope. Consistently, in the opposite direction, Figure S4 depicts that exposure of the myocytes to exogenous ROS (100 μM hydrogen peroxide; H_2O_2) induced a negative inotropic effect accompanied with a decrease in Ca^{2+} transient amplitude and a desensitization of contractile myofilaments to Ca^{2+}. These data are supported by a previous report [22]. Taken together, these results suggest that ROS-induced p-38K activation

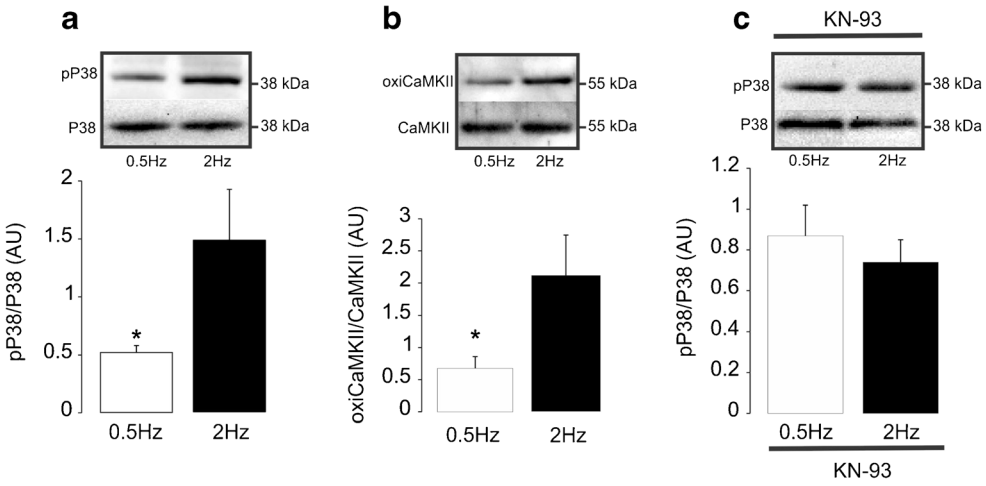

Fig. 5 Activation of kinases during the increase in stimulation frequency. Representative WB and average data of the phosphorylation of p-38K (5 rats, $n = 5$; **a**) or the oxidation of CaMKII (5 rats, $n = 5$; **b**) when the heart was stimulated at 0.5 or 2 Hz during 1 min. **c** shows that the pre-incubation of the hearts with KN-93 prevented the phosphorylation of p-38K (5 rats, $n = 5$), indicating the sequence of the pathway during the increase in stimulation frequency. Asterisk indicates $p < 0.05$ vs 2 Hz

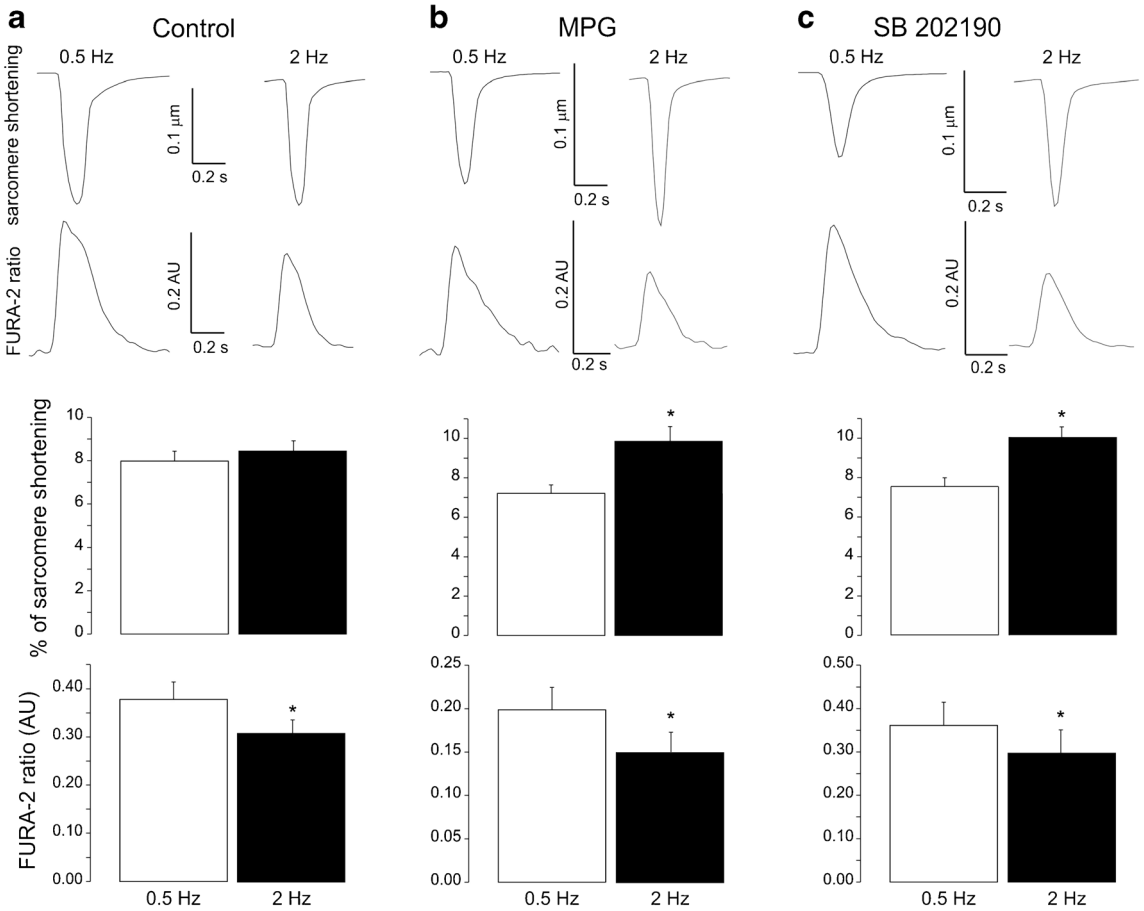

Fig. 6 Correlation between sarcomere length shortening and calcium transients in myocytes loaded with Fura-2. Upper panels show representative twitches of sarcomere shortening and calcium transient in the same cell and at the same time in control (**a**), in the presence of MPG (**b**), or in the presence of SB-202190 (**c**). Lower panels illustrate the average data of the three previous situations, showing that under control condition (6 rats, $n = 10$), the cells exhibit a decrease in calcium transient with a flattened FFR, whereas in the presence of MPG (4 rats, $n = 8$) or SB-202190 (4 rats, $n = 8$), the cells exhibit a decrease in calcium transients accompanied with a clear positive FFR. Asterisk indicates $p < 0.05$ vs 0.5 Hz

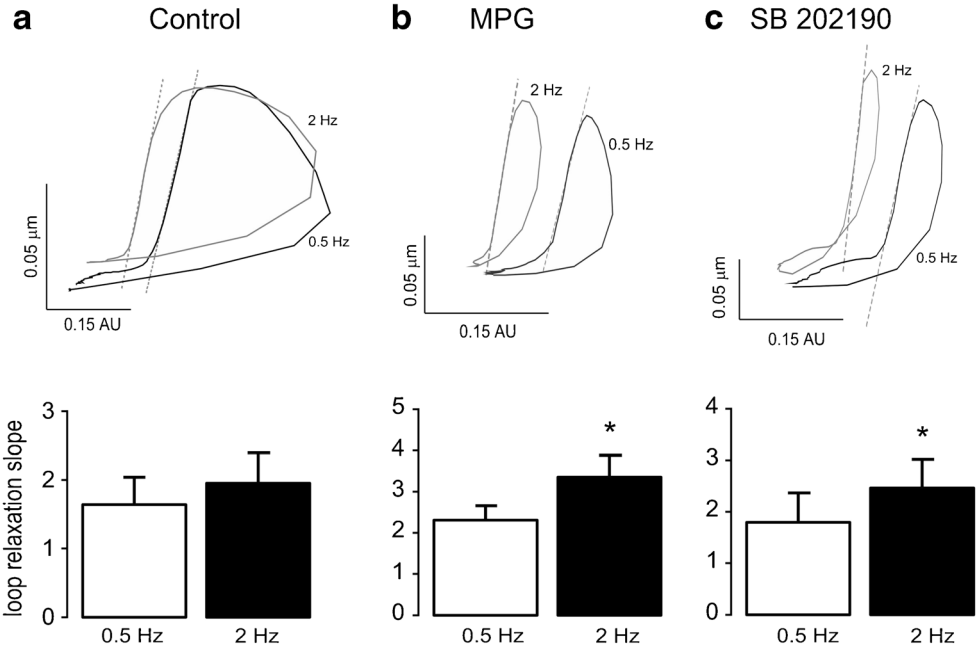

Fig. 7 SL shortening-free Fura-2 and Fura-2 bound calcium ratio loops showing changes in myofilament calcium sensitivity upon increasing stimulation frequency. Representative loops (upper panels) and average data of the loop relaxation slope (lower panels) from control (**a**, 6 rats, $n = 10$), MPG-treated (**b**, 4 rats, $n = 8$), and SB-202190-treated cardiomyocytes (**c**, 4 rats, $n = 8$) at 0.5 and 2 Hz are shown. Dotted lines represent the slope obtained from differentiation of the k point of the relaxation phase (see "Materials and methods" section). Asterisk indicates $p < 0.05$ vs 0.5 Hz

represents a desensitizing mechanism triggered by the increase in stimulation frequency, which likely contributes to the typical negative or flat FFR observed in rat myocardium.

Discussion

The results presented herein showed a negative FFR in the intact and BCECF-loaded isolated rat ventricular myocytes and a flat FFR in the Fura-2-loaded ones. In agreement with our results, several authors have demonstrated a negative FFR in rat [31–33, 49]. However, the intracellular mechanism implicated in such phenomenon is not fully understood. Herein, the negative/flat FFR was associated with a parallel decline in the intracellular Ca^{2+} transient. In this regard, other studies have proposed that the negative staircase in rat ventricle might be due to progressive refractoriness in the SR Ca^{2+} release process at higher stimulation frequency [3, 6]. In addition, the high intracellular Na^{+} level and/or the SR Ca^{2+} overload reported in the literature for rat cardiomyocytes can also explain this phenomenon [33, 35, 38].

The stimulation frequency employed in the present work (0.5–3 Hz) is the widely used pacing rate for the isolated rat cardiomyocytes. Higher stimulation frequency would induce incomplete relaxation. However, these range of frequencies are far lower than the physiological heart frequency of the rat, which is in the order of 5–20 Hz. Nevertheless, although we recognized the limitation of these experiments to reflect the pure physiological status of the rat heart, it is important to note that the negative FFR is still present when cardiac contractility of rat hearts is studied in intact hearts at normal (high) frequencies [37, 58].

The increase in the frequency of stimulation in cat ventricular papillary muscle was shown to induce a decrease of intracellular pH (pH_i) in an extracellular free HCO_3^- solution (HEPES) [10]. Since H^+ competes with Ca^{2+} for the binding to contractile proteins, the intracellular acidosis might also explain the reduction in contractile force. In contrast, it was also demonstrated that in the presence of extracellular HCO_3^-, the increase in the frequency of stimulation generates an increase in pH_i [10]. However, in the present study, working with rat cardiomyocytes and extracellular HCO_3^-, we did not detect changes in pH_i during the increase in pacing rate. Therefore, under the present experimental conditions, pH-dependent mechanisms are not involved in the decrease in contractility induced by the increase in the frequency of stimulation.

The negative/flat FFR was converted in a positive one in the presence of the ROS scavenger, MPG. Similar results were obtained in the presence of the NOX blocker, apocynin, or the mitoK_{ATP} blocker, 5-HD. These data suggest that ROS are involved in the negative staircase of the rat myocyte. The NOX [4] and the mitochondrion are the most important sources of ROS in the cardiac cell. Moreover, a crosstalk between them has been described [18], so it might be possible that the increase in the frequency of stimulation induces an increase in mitochondrial ROS production, which in turn stimulates NOX to produce more ROS. These results are in agreement with several studies that showed that the increase in the frequency of stimulation induces an increment in ROS production [24, 50].

Since ROS were involved in the negative/flat FFR detected in rat cardiomyocytes, we evaluated the role of the redox-sensitive kinases p-38K and CaMKII in this mechanism. The pre-incubation of myocytes with SB-202190 (p-38K blocker) or KN-93 (CaMKII blocker) induced a positive FFR. Furthermore, we found that under our conditions, there was a rise in the oxidation level of CaMKII and in the phosphorylation level of p-38K at 2 Hz in comparison to 0.5 Hz, suggesting that these kinases increase their activity during the higher frequency. Moreover, the phosphorylation of p-38K at 2 Hz was prevented with KN-93, indicating that the sequence of events in the intracellular pathway is ROS production-CaMKII oxidation-p-38K phosphorylation.

Molecular mechanisms that activate ROS production and CaMKII oxidation are frequently related to contractile dysfunction in disease hearts. However, in the present work, these mechanisms are involved in the regulation of contractile function in healthy myocytes. The role of the reactive oxygen species (ROS) as intracellular signaling molecules which regulate physiological process as pH_i or myocyte contractility has been well investigated. It has been demonstrated that ROS increase the phosphorylation of several kinases as ERK 1/2 [48, 51] or AKT [16], which in turn can modulate the activity of alkalinizing mechanisms [14, 15, 23]. Moreover, ROS have been shown to participate in angiotensin II-, endothelin-1-, and aldosterone-induced positive inotropic effect [8, 12, 57]. On the other hand, CaMKII is a kinase mainly involved in several pathological processes as arrhythmias during ischemia/reperfusion, hypertrophy, or heart failure. However, it is also true that CaMKII participates in the physiological regulation of proteins involved in calcium management as the L-type calcium channels [30, 42]. Moreover, CaMKII has been proposed to be involved in the control of heart frequency in sinoatrial node [27]. It could seem a contradiction the fact that in terms of contractility, it has been previously reported that ROS production and CaMKII activation are involved in the blunted FFR during heart failure [52, 54] while in the data presented herein, the same protagonists are suggested to play the same role but in a physiological process, like the normal FFR of the rat. Although the explanation of this matter is not apparent to us, we could speculate that it is possible that the effects observed in the normal rat myocardium represent the reflection of the consequences of the exacerbation of these mechanisms in other species.

As previously reported, the increase in the stimulation frequency of rat cardiomyocytes was associated with a decrease in the amplitude of the Ca^{2+} transient [29, 32]. This phenomenon was also observed when a ROS scavenger or a p-38K blocker was included in the extracellular solution, situations in which a clear positive inotropic effect was detected. Thus, although direct effects of these drugs cannot be completely discarded, a myofilament Ca^{2+}-sensitizing effect was evident when the redox-sensitive activation of p-38K was inhibited. This mechanism was corroborated by the analysis of the sarcomere length shortening/calcium level loops. On the other hand, in the control Fura-2-loaded cells, despite the fact that the amplitude of the Ca^{2+} transient was diminished upon the increase in the stimulation frequency, the contractility was unaltered. This phenomenon could be explained by the presence of a sensitizing effect which opposes to the p-38K-mediated desensitizing one. The mechanistic and molecular nature of this sensitizing effect, which is unmasked when the p-38K pathway is blocked, was not elucidated in the present work, representing an interesting question to be answered with future investigations.

Several mechanisms could be involved in the p-38K-mediated decrease in myofilament responsiveness to Ca^{2+}. Among these, a modulation of pH_i and/or a change in the phosphorylation status of contractile proteins seem the most probable candidates. However, in the present study, we failed to observe changes in pH_i during the increase in the frequency of stimulation. Therefore, a reversible modification of the contractile apparatus could represent a more likely scenario. Phosphorylation of troponin I (TnI) is known to reduce myofilament responsiveness to Ca^{2+}. However, it was demonstrated that p-38K is unable to phosphorylate this protein, indicating that TnI is not a direct downstream target of p-38K [28]. Another study has indicated that heat shock proteins (HSP) such as HSP27 are activated by p-38K, causing their translocation to the sarcomeric Z line, where they lead to a decrease in myosin ATPase activity and contractile depression, possibly through modifications in sarcomeric scaffolding proteins such as α-actin [11]. Nevertheless, the identification of the molecular mechanisms that are responsible for the ROS-p-38K-mediated decrease in myofilament responsiveness requires further investigation.

The exclusive use of a pharmacological approach is an important limitation of the present work. In order to minimize this limitation, we used different drugs to probe the participation of ROS (MPG, Apo, and 5-HD). In addition, we have performed experiments with H_2O_2 to mimic the effects of the increase in pacing rate. Moreover, in order to address the effects of the kinases, we did both functional and western blot analyses and we found a positive correlation. Nevertheless, the requirement of molecular tool employment to confirm these results is recognized.

In summary, we have presented evidence indicating for the first time that a phenomenon of desensitization of the contractile proteins contributes to the negative/flat FFR in rat ventricular myocytes. Moreover, we have demonstrated that during the increase in stimulation frequency, there occurs an activation of a pathway that involves ROS production, CaMKII oxidation, and p-38K phosphorylation, culminating in a decrease in myofilament responsiveness to Ca^{2+} and a negative inotropic effect. The potential association of these findings to the typical negative or flat FFR observed in the human failing heart where ROS production and CaMKII and p-38K activity have been shown to be increased [2, 5, 7, 21, 25, 34, 39] represents an interesting idea that deserves future investigation.

Compliance with ethical standards All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of La Plata University.

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