Mitochondrial and cytosolic calcium in rat hearts under high-K^+ cardioplegia and pyruvate: mechano-energetic performance

A.E. Consolini, M.I. Ragone, and P. Bonazzola

Abstract: High-K^+-cardioplegia (CPG) and pyruvate (Pyr) are used as cardioprotective agents. Considering that mitochondria play a critical role in cardiac dysfunction, we investigated the effect of CPG on mitochondrial Ca^{2+} uptake and sarcoreticular (SR) calcium handling. Cytosolic and mitochondrial Ca^{2+}, as well as mitochondrial membrane potential ($\Delta\Psi m$) were assessed in rat cardiomyocytes by confocal microscopy. Mechano-calorimetrical correlation was studied in perfused hearts. CPG did not modify JC-1 ($\Delta\Psi m$), but transiently increased, by up to 1.8 times, the Fura-2 (intracellular Ca concentration, $[Ca^{2+}]i$) and Rhod-2 (mitochondrial free Ca concentration $[Ca^{2+}]m$) fluorescence of resting cells, with exponential decays. The addition of 5 $\mu mol \cdot L^{-1}$ thapsigargin (Tpg) increased the Rhod-2 fluorescence in a group of cells without any effect on the Fura-2 signal. In rat hearts perfused with CPG, 1 $\mu mol \cdot L^{-1}$ Tpg decreased resting heat rate (ΔH_r: -0.44 ± 0.07 $mW \cdot g^{-1}$), while the addition of 5 $\mu mol \cdot L^{-1}$ KB-R7943 increased resting pressure (ΔrLVP by $+5.26 \pm 1.10$ mm Hg; 1 mm Hg = 133.322 Pa). The addition of 10 $mmol \cdot L^{-1}$ Pyr to CPG increased H_r ($+3.30 \pm 0.24$ $mW \cdot g^{-1}$) and ΔrLVP ($+2.2 \pm 0.4$ mm Hg), which are effects potentiated by KB-R7943. The results suggest that under CPG, (*i*) there was an increase in $[Ca^{2+}]i$ and $[Ca^{2+}]m$ (without changing $\Delta\Psi m$) that decayed by exothermic removal mechanisms; (*ii*) mitochondrial Ca^{2+} uptake contributed to the removal of cytosolic Ca^{2+}, in a process that was potentiated by inhibition of sarco–endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and reduced by KB-R7943; (*iii*) under these conditions, SERCA represents the main energetic consumer; (*iv*) Pyr increased the energetic performance of hearts,mainly by inducing mitochondrial metabolism.

Key words: Ca^{2+}, calorimetry, heart, cardiomyocytes, cardioplegia, Fura-2, Rhod-2, pyruvate.

Résumé : La cardioplégie riche (CPG) en K^+ et le pyruvate (Pyr) sont utilisés comme agents cardioprotecteurs. Considérant que les mitochondries jouent un rôle important dans la dysfonction cardiaque, nous avons examiné l'effet de la CPG sur la capture de Ca^{2+} mitochondrial et l'homéostasie du calcium du RS. Nous avons évalué par microscopie confocale le Ca^{2+} cytosolique et mitochondrial ainsi que le potentiel membranaire mitochondrial ($\Delta\Psi m$) dans les cardiomyocytes de rats. Nous avons examiné la corrélation mécano-calorimétrique dans les cœurs perfusés. La CPG n'a pas modifié la fluorescence du JC-1 ($\Delta\Psi m$), mais a augmenté transitoirement jusqu'à 1,8 fois celle du Fura-2 ($[Ca^{2+}]_i$) et du Rhod-2 ($[Ca^{2+}]_m$) des cellules au repos, avec des diminutions exponentielles. L'addition de 5 $\mu mol \cdot L^{-1}$ de thapsigargine (Tpg) a augmenté la fluorescence du Rhod-2 dans un groupe de cellules, sans influer sur le signal Fura-2. Dans les cœurs perfusés avec la CPG, 1 $\mu mol \cdot L^{-1}$ de Tpg a diminué la fréquence cardiaque au repos (ΔH_r : $-0{,}44 \pm 0{,}07$ $mW \cdot g^{-1}$), alors que l'ajout de 5 $\mu mol \cdot L^{-1}$ de KB-R7943 a augmenté la pression au repos (ΔrPVG de $+5{,}26 \pm 1{,}10$ mm Hg. L'ajout de 10 $mmol \cdot L^{-1}$ de Pyr à la CPG a entraîné une augmentation de la H_r ($+3{,}30 \pm 0{,}24$ $mW \cdot g^{-1}$) et de la ΔrPVG ($+2{,}2 \pm 0{,}4$ mm Hg), un effet qui a été potentialisé par KB-R7943. Les résultats donnent à penser que durant la perfusion avec CPG : (*i*) il y a eu une augmentation des ($[Ca^{2+}]_i$ et ($[Ca^{2+}]_m$ (sans modification du $\Delta\Psi m$), qui ont été diminuées par des mécanismes d'élimination exothermiques; (*ii*) la capture de Ca^{2+} mitochondrial a contribué à l'élimination du Ca^{2+} cytosolique, dans un processus qui a été potentialisé par l'inhibition de la SERCA, et elle a été diminuée par KB-R7943; (*iii*) la SERCA représente le principal consommateur d'énergie dans ces conditions; (*iv*) le Pyr a augmenté la performance énergétique des cœurs, principalement en induisant le métabolisme mitochondrial.

Mots-clés : Ca^{2+}, calorimétrie, cœur, cardiomyocytes, cardioplégie, Fura-2, Rhod-2, pyruvate.

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A.E. Consolini. Cátedra de Farmacología, Dept. Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), 47 y 115 s/n (1900) La Plata, Argentina; Instituto de Recerca, Cardiología, Hospital Vall d'Hebron, Barcelona, Spain.
M.I. Ragone. Cátedra de Farmacología, Dept. Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), 47 y 115 s/n (1900) La Plata, Argentina.
P. Bonazzola. Instituto de Investigaciones Cardiológicas, Facultad de Medicina, Universidad de Buenos Aires (UBA)-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

Corresponding author: Alicia E. Consolini (e-mail: dinamia@biol.unlp.edu.ar).

Introduction

Mitochondria play a critical role in the regulation of the cytosolic Ca^{2+} concentration in different cell types, including cardiomyocytes, both under physiological and pathological conditions (Rizzuto et al. 2000). Mitochondrial Ca^{2+} transients have been observed in rat cardiomyocytes, which follow the time course of cytosolic transients (Bell et al. 2006). Further, during transient ischemia, mitochondria contribute to the reduction of cytosolic Ca^{2+} overload, provided that the mitochondrial membrane potential ($\Delta\Psi m$) is not completely dissipated (Ruiz-Meana et al. 2007). Upon cardiac reperfusion, mitochondria may induce cell death by releasing Ca^{2+} through the open mitochondrial transition pore (MTP) and the concomitant energetic failure (Halestrap 2009). Rapid sarcorreticular (SR) Ca^{2+} oscillations may promote MTP opening in regions of close anatomical interaction between both organelles (Ruiz-Meana et al. 2009). Nevertheless, depending on the severity of the injury, mitochondria could also have a protective role by accumulating large amounts of Ca^{2+} without inducing the MTP (Griffiths 2009).

Cardioplegic solutions contribute to prevent ischemic-reperfusion injury in different animal species (Stowe et al. 2000; Wang et al. 2003). These solutions contain high extracellular K concentration ($[K^+]o$), with different $[Ca^{2+}]o$, and sometimes $[Mg^{2+}]o$ (Mei et al. 2006). Cardioplegic arrest of the beating heart has energetic consequences, such as a rapid reduction in oxygen consumption owing to the fall in cardiac demand. Nevertheless, the presence of high-$[K^+]o$ increased basal oxygen consumption (Siess 1987) and basal heat rate (H_r) in rat papillary muscle (Holroyd et al. 1990) and rat whole hearts (Márquez et al. 1997), both of which depend on ionic pump activity and mitochondrial metabolism (Loiselle 1987). Accordingly, there are reports indicating that the increase in intracellular Ca concentration ($[Ca^{2+}]i$) caused by high-$[K^+]o$-treatment, is sensitive to verapamil (Powell et al. 1984), as is the basal heat rate (H_r) (Márquez et al. 1997). Despite the fact that high-K^+-cardioplegia promotes a voltage-dependent Ca^{2+} influx during cardiac arrest, this maneuver is cardioprotective when given before the induction of ischemia-reperfusion (I/R) in rat hearts, by preventing diastolic dysfunction and increasing contractile recovery, in part by Ca^{2+} removal through the sarcolemmal Na^+/Ca^{2+}-exchanger (SL-NCX) (Consolini et al. 2004). Mitochondria also play a protective role, since stunning was induced by pharmacological inhibition of uniporter with Ru-360 and mitochondrial Na/Ca-exchanger (mNCX) with clonazepam (Consolini et al. 2007; Ragone and Consolini 2009). Therefore, our hypothesis was that cardioplegia (CPG) could favor the participation of mitochondria in the regulation of the cytosolic and SR Ca^{2+} during reperfusion.

On the other hand, addition of the aerobic metabolic substrate pyruvate (Pyr) during and (or) after cardioplegic arrest has been shown to be cardioprotective by attenuating oxidative stress and preserving mitochondrial enzymes (Knott et al. 2006). This, however, has a dual effect on cardiac SR Ca^{2+} release (Zima et al. 2003). Despite the fact that Pyr has also a cardioprotective effect in patients (Olivencia-Yurvati et al. 2003), there is no evidence to suggest that this effect is mediated by Ca^{2+} transfer between mitochondria and the sarcoplasmic reticulum.

Thus, we investigated the functional interaction between the sarcoplasmic reticulum and mitochondria with regards to Ca^{2+} handling in rat cardiac myocytes exposed to CPG. We further explored the mechano-calorimetrical consequences of this procedure in isolated rat hearts arrested by CPG, in the presence or absence of Pyr. Our results indicate that CPG induced a raise in mitochondrial Ca^{2+}, with a slow decay, which was increased after inhibition of sarco–endoplasmic reticulum Ca^{2+}-ATPase (SERCA). In hearts, SERCA inhibition also increased the diastolic resting tension, but reduced the net energetic expenditure. In contrast, the addition of 10 $mmol \cdot L^{-1}$ Pyr to CPG increased the energetic performance, with a slight diastolic contracture. Our results suggest that CPG induces Ca^{2+} transfer between mitochondria and the sarcoplasmic reticulum, and that the Pyr effect would mainly be associated with an increase in mitochondrial metabolism.

Methods

Our studies followed the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH 1996), and our methods were reviewed and approved by the respective animal care committees of the Vall d'Hebron Hospital, the Universidad Nacional de La Plata, and the Facultad de Medicina Universidad de Buenos Aires.

Confocal microscopy in rat cardiomyocytes

Isolation of cardiac myocytes

Hearts from adult Wistar rats (300 g weight) were perfused in a Langendorff system with a modified Krebs solution virtually free of Ca^{2+} (in $mmol \cdot L^{-1}$: 110 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 dextrose; pH 7.4), also containing 0.03% type II collagenase (Serba), as previously described (Ruiz-Meana et al. 1999). Rod-shaped cardiac myocytes were selected from the dissociated tissue by a series of differential centrifugations and progressive normalization of $[Ca^{2+}]o$.

Confocal microscopy

Isolated cardiomyocytes were plated onto laminin-precoated glass-bottom culture dishes, and incubated at room temperature (about 25 °C) with either 5 $\mu mol \cdot L^{-1}$ Fura-2 acetoxymethyl ester (AM) for 45 min (to analyze cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$)), 4 $\mu mol \cdot L^{-1}$ Rhod-2 AM for 60 min (to analyze mitochondrial free Ca^{2+} concentration, ($[Ca^{2+}]m$)), or 10 $\mu mol \cdot L^{-1}$ JC-1 for 15 min (to analyze changes in $\Delta\Psi m$) (Molecular Probes, USA). Loaded resting cells were exposed to 400 µL of a Krebs solution (in $mmol \cdot L^{-1}$: 1.2 $MgSO_4$, 130 NaCl, 1.2 KH_2PO_4, 3.6 KCl, 20 Hepes, 1 $CaCl_2$, and 5 dextrose) for 2 min, and then a pulse of 16 µL of 0.5 $mol \cdot L^{-1}$ KCl was added to obtain a high-K^+ (25 $mmol \cdot L^{-1}$) low-Ca^{2+} (1 $mmol \cdot L^{-1}$) cardioplegia (CPG). In a subset of experiments, 5 $\mu mol \cdot L^{-1}$ thapsigargin (Tpg) was added 3 min after cardioplegic solution, to specifically block SR Ca^{2+} uptake. Fluorescence changes were monitored for either 15 min (Fura-2 loaded cells) or 20 min (Rhod-2 loaded cells). JC-1 loaded cells were exposed to Krebs solution for 1 min, and to CPG for an additional 5 min, and changes in fluorescence were monitored throughout the whole period (5 min). Despite the increase

in [Ca^{2+}]c, cells did not develop contracture, possibly owing to the presence of a high-[K^+]o, except for 3 isolated cells (2 with Fura-2 and 1 with Rhod-2), which were not included in the analysis.

To evaluate [Ca^{2+}]c, Fura-2 loaded cells were alternatively excited at 340 and 380 nm by means of a fast speed monochromator, every 1 s for 15 min. Changes in fluorescence emission at 510 nm from previously defined areas in each cell were monitored over time using an intensified digital camera (CCD) set on an Olympus IX70 (×40, NA 1.4). Fluorescence data were subsequently analyzed using a commercially available software (QC2000, Visitech, UK). Results were expressed as the ratio of 340:380 nm emission fluorescence intensities with respect to the initial value.

To investigate [Ca^{2+}]m, Rhod-2 loaded cells were excited at 568 nm, every 5 s for 20 min, using a confocal Ar–Kr laser system (Yokogawa CSU10, Nipkow spinning disk) mounted on a fluorescence inverted microscope (Olympus IX70, ×60, NA 1.4). Changes in fluorescence emission (>590 nm) from previously defined areas in each cell were monitored over time, using a commercially available software (VoxCell scan, Visitech, UK). Results were expressed as relative fluorescence intensity with respect to the initial value.

To analyze $\Delta\Psi$m, JC-1 loaded cells were excited at 488 nm, every 10 s for 5 min, using the same confocal Ar–Kr laser system (×60, NA 1.4). Relative changes in $\Delta\Psi$m were estimated from fluorescence emission ratios at 590 nm/520 nm. A positive control for JC-1 was obtained by adding 200 μmol·L^{-1} dinitrophenol (DNP) to a control cell. Results were expressed as changes in the ratio of fluorescence intensity with respect to the initial value.

Calorimetry of isolated rat hearts

Experimental protocols in perfused rat hearts

Wistar rats (250–300 g weight) were anesthetized with an overdose of pentobarbital (60–70 mg·kg^{-1}), and after quick excision, the heart was retrogradely perfused with Krebs solution (in mmol·L^{-1}: 1 $MgCl_2$, 125 NaCl, 0.5 NaH_2PO_4, 7 KCl, 25 $NaHCO_3$, 1 $CaCl_2$, and 6 dextrose; bubbled with 95% O_2 – 5% CO_2) by Langerdorff technique, at a constant flow of 6 mL·min^{-1}·g^{-1} (Ponce-Hornos et al. 1995). The [K^+]o was maintained at 7 mmol·L^{-1} to avoid spontaneous heart beating when measuring H_r before adding CPG. Both atria were removed and a little cut was done in the interventricular septum close to the aorta to eliminate spontaneous beating. A latex balloon was placed in the left ventricle and connected by a flexible cannula to a Gould Statham P23db pressure transducer (Gould Statham Instruments, Inc., Puerto Rico). While continuously perfused, the heart was placed into the calorimetric chamber, which was closed and submerged in a bath kept at a controlled temperature (±0.01 °C) as previously described (Consolini et al. 2007). The heart was stabilized under electrical stimulation at 1 Hz, 5 V-5 msec, by means of 2 electrodes inside the chamber, which were connected to a Grass SD9 electrical stimulator (Grass Instruments, USA). Total heat rate (H_t) and left intraventricular pressure (LVP) at optimal volume were continuously recorded.

In the first series of experiments, hearts were exposed to conditions identical to those used with the isolated cardiomyocytes (at 25 °C and CPG+1 mmol·L^{-1} Ca^{2+}). After a stabilization period of at least 60 min with Krebs solution and stimulation at 1 Hz, hearts were progressively subjected, for a 15 min period, to the following protocols: (*i*) perfusion with CPG+1 mmol·L^{-1} Ca^{2+}; (*ii*) perfusion with CPG+1 mmol·L^{-1} Ca^{2+} + 1 μmol·L^{-1} Tpg (to block the SERCA); and (*iii*) perfusion with CPG+1 mmol·L^{-1} Ca^{2+} + 1 μmol·L^{-1} Tpg + 5 μmol·L^{-1} KB-R7943 (to simultaneously block SERCA, the reverse mode of SL-NCX, and mitochondrial Ca^{2+}-uniporter) (Santo-Domingo et al. 2007).

To evaluate the effects of the cardioplegic media, as well as the mechanism by which a high-[K^+]o triggers Ca^{2+} influx, another series of 5 hearts, beating at 1 Hz in Krebs-C at 25 °C, were subsequently exposed to the following treatments for about 15 min: (*i*) resting condition created by the absence of stimulation; (*ii*) cardioplegia with (*a*) 25 mmol·L^{-1} K+0.5 mmol·L^{-1} Ca, and (*b*) 25 mmol·L^{-1} K+1 mmol·L^{-1} Ca; (*iii*) reversion to Krebs-C and steady stimulation, (*iv*) 25 mmol·L^{-1} K+1 mmol·L^{-1} Ca; and finally, (*v*) 25 mmol·L^{-1} K+1 mmol·L^{-1} Ca + 10 μmol·L^{-1} nifedipine (Nif). Another group of 5 hearts, in a resting state in Krebs-C, was exposed to the following sequence of treatments: (*i*) cardioplegia with 25 mmol·L^{-1} K+1 mmol·L^{-1} Ca for 15 min; (*ii*) reversion to Krebs-C in a resting state; (*iii*) addition of 2 μmol·L^{-1} tetrodotoxin (TTX), and then (*iv*) 25 mmol·L^{-1} K + 1 mmol·L^{-1} Ca + 2 μmol·L^{-1} TTX.

In another series of experiments, hearts were perfused at 30 °C with Krebs solution and stimulated at 1 Hz. Hearts were then perfused for 15 min with the previously described cardioprotective cardioplegia (CPG+0.5 mmol·L^{-1} Ca^{2+}) (Consolini et al. 2004). This was followed by any one of the following protocols: (*i*) perfusion with CPG+0.5 mmol·L^{-1} Ca^{2+} + 10 mmol·L^{-1} pyruvate (Pyr); (*ii*) perfusion with CPG+0.5 mmol·L^{-1} Ca^{2+} + 10 mmol·L^{-1} pyruvate (Pyr) + 5 μmol·L^{-1} KB-R7943 ; (*iii*) perfusion with CPG+0.5 mmol·L^{-1} Ca^{2+} + 5 μmol·L^{-1} KB-R7943.

Calorimetric measurements

To measure heat release by the perfused heart (heat rate, *H*), a home-made calorimeter was used following the methods of Ponce-Hornos et al. (1995). The calorimeter consisted of a copper mass with an internal chamber where 2 ceramic modules (Melcor Thermoelectrics, USA), containing 127 thermosensitive units each, were placed. These units sense the temperature changes between the internal and the external copper mass, which is submerged and equilibrated with the constant-temperature bath. It is also possible to measure the heat rate before and after the introduction of the heart, as a baseline, in the presence and absence of perfusion. Calibration of the calorimeter was conducted by injecting a constant electrical power of 2 mW through the muscle acting as an electrical impedance inside the chamber. A calibration factor (in mW·V^{-1}) was obtained in the presence of perfusion, which was used to calculate the muscle H_t during steady-state beating of the heart, and H_r when the heart was arrested by either stopping the electrical stimulation or by perfusing with CPG. The signals for heat rate and intraventricular pressure coming from the calorimeter and the pressure transducer, respectively, were amplified and recorded on a Grass model 7 polygraph (Grass Instruments) and digitalized through a A/D converter (TL-1 DMA; Axon Instruments, USA). Results for H_t or H_r were expressed in mW·g^{-1} of wet heart weight.

Drugs

The drugs and the concentrations used were as follows: 5 μmol·L^{-1} Fura-2 AM, 4 μmol·L^{-1} Rhod-2 AM, and 10 μmol·L^{-1} JC-1 (Molecular Probes, USA); 5 μmol·L^{-1} Tpg in cells, and 1 μmol·L^{-1} in hearts (Calbiochem, USA); 5 μmol·L^{-1} KB-R7943 (Calbiochem, USA); 10 μmol·L^{-1} Nif (Sigma-Aldrich, USA); 2 μmol·L^{-1} tetrodotoxin (TTX) (Sigma-Aldrich, USA).

Mathematical and statistical analysis

Where indicated, the changes in fluorescence versus time for the Rhod-2 and Fura-2 responses were analyzed in their decay period (from 200 s of recording to the finish) by non-linear fitting, using Origin 6.0 software. The extracted parameters are presented as mean ± SEM (n = number of experiments analyzed), for all of the results. Unpaired Student t tests were used for comparing the fitting parameters of the relative fluorescence curves obtained with and without Tpg. In each whole-heart experiment, a linear fit was done to evaluate the rate of increase inchange in resting left ventricular pressure (ΔrLVP) under either CPG, CPG+Tpg, or CPG+Tpg+KB-R7943. One-way ANOVA was applied for comparison of the observed slopes from repeated observations. The t test was used for determining whether the ΔH_r values were different from 0 in Fig. 4*c*. One-way ANOVA followed by a posteriori Tukey's tests were used for comparing ΔrLVP and ΔH_r values at 15, 30, and 45 min for matched observations (Fig. 4). The 2-way ANOVA was applied for comparing either ΔrLVP or H_r values with time among the 3 CPG treatments in Fig. 6, followed by a posteriori Bonferroni tests. Statistical tests were performed by the Graph Pad Prism 4 software. A significance level of $p < 0.05$ was used in all cases.

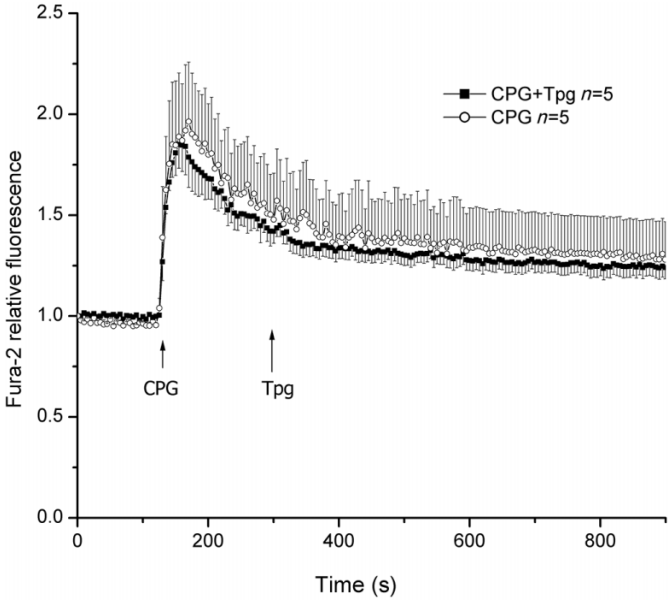
Results

Effect of high-K$^+$ cardioplegia on cytosolic and mitochondrial Ca^{2+} concentration

Isolated cardiomyocytes exposed to high-K$^+$-CPG (25 mmol·L^{-1} K$^+$+1 mmol·L^{-1} Ca^{2+}) experienced a quick and pronounced increase in the fluorescence ratio for Fura-2 cells, up to 1.96 ± 0.29 (n = 5), reflecting a rise in [Ca^{2+}]c (Fig. 1). This peak was followed by a slow decay during the next 10 min, which was fitted (from 80 s after CPG addition) to a single exponential function with an asymptotical suprabasal fluorescence ratio of 1.26 ± 0.15 (n = 5) (Table 1). Addition of 5 μmol·L^{-1} Tpg after CPG did not significantly change the parameters of exponential decay of fluorescence (Fig. 1 and Table 1).

The relative intensity of Rhod-2 fluorescence in cardiomyocytes quickly and significantly increased to a maximum of 1.89 ± 0.17 (n = 7) after exposure to CPG, reflecting a rapid rise in [Ca^{2+}]m. This peak was followed by a slow and sustained decay (Fig. 2A), which was also fitted to a single exponential function with an asymptotical suprabasal fluorescence ratio of 1.20 ± 0.11 (n = 7) (Table 2). In contrast to the absence of effect on the cytosolic Ca^{2+} decay (Fura-2), the addition of 5 μmol·L^{-1} Tpg after CPG showed 2 different mitochondrial Ca^{2+} reactions: in 45% of cells (5 out of 11 cells) Tpg did not modify mitochondrial Ca^{2+} exponential decay (Table 2), while in the remaining 54% (6 out of 11 cells)

Fig. 1. Changes in the cytosolic Ca^{2+} signal estimated by the 340:380 nm Fura-2 fluorescence ratio over time in rat cardiomyocytes, before and after adding cardioplegia (CPG, 25 mmol·L^{-1} K$^+$ + 1 mmol·L^{-1} Ca^{2+}, at the arrow) in two groups of cells, one with only CPG (open circles, n = 5) and another in which 5 μmol·L^{-1} thapsigargin was added to CPG (at the arrow, Tpg, filled squares, n = 5). Results are expressed as mean ± SEM.



the relative Rhod-2 fluorescence further increased up to 2.25 ± 0.48 (n = 6), always without changes in cell length (Fig. 2B, $p < 0.001$ compared with cells without Tpg).

In addition, the relative red:green intensity of JC-1 fluorescence was not significantly affected by CPG, indicating that ΔΨm did not change (Fig. 3). JC-1 fluorescence caused subsarcolemmal mitochondria to appear red, suggesting they were more polarized than mitochondria inside the cell, which appeared green (Fig. 3A, Web version only). The uncoupling agent DNP 200 μmol·L^{-1} induced both a drastic change from red to green fluorescence in the whole cell, denoting dissipation of the proton gradient, and a cell shortening due to rigor (Fig. 3D, Web version only).

Inhibition of SERCA and mitochondrial Ca^{2+} uniporter in cardioplegic hearts

Treatment of isolated perfused rat hearts with CPG (Krebs with 25 mmol·L^{-1} K$^+$ + 1 mmol·L^{-1} Ca^{2+}) induced cardiac arrest. Figure 4A shows two individual recordings of H_t in which it can be seen that when perfusing CPG, a beating heart decreases the H_t to H_r. However, a previously arrested heart showed an increase in H_r in a transient state, followed by a steady state. Resting for 15 min increased intraventricular pressure (ΔrLVP) up to +6.4 ± 1.3 mm Hg (n = 9) above the pre-CPG level (Fig. 4B). The average rate of increase in ΔrLVP calculated for the last 10 min in CPG was +0.151 ± 0.045 mm Hg·min^{-1} (n = 9). Simultaneously, the total heat rate of beating hearts decreased to the resting heat-rate value (H_r) of 5.85 ± 0.52 mW·g^{-1} (n = 9). Addition of 1 μmol·L^{-1} Tpg induced a further increase in rLVP up to +2.92 ± 0.80 mm Hg (n = 9) above the CPG level, with a rate of increase of 0.199 ± 0.053 mm Hg·min^{-1} (n = 9). Simultane-

Table 1. Parameters of the exponential decay fitted from 200 s recording to the end of the Fura-2 signal curve obtained in cardiomyocytes treated with cardioplegia (CPG) with thapsigargin (CPG+Tpg) and withoutTpg, as shown in Fig. 1.

Parameters	CPG (n = 5)	CPG+Tpg (n = 5)	Student t test
y_o (asyntota)	1.26±0.15	1.25±0.05	p = 0.95
A_1	2.78±0.85	2.24±0.60	p = 0.61
t_c (s)	106.8±9.22	111.8±29.6	p = 0.87

Note: t_C is the time constant; $y = y_o + A_1\exp[-t\cdot(t_c)^{-1}]$.

Table 2. Parameters of the exponential decay fitted from 200 s of recording to the end of the Rhod-2 signal curve obtained in cardiomyocytes treated with cardioplegia (CPG), and in the cells in which the further addition of thapsigargin did not increase fluorescence (CPG+Tpg), as shown in Fig. 2A.

Parameters	CPG (n = 7)	CPG+Tpg (n = 5)	Student t test
y_o (asyntota)	1.20±0.11	0.98±0.05	p = 0.14
A_1	1.53±0.33	0.72±0.10	p = 0.073
t_c (s)	244.3±24.56	354.7±91.27	p = 0.203

Note: t_C is the time constant; $y = y_o + A_1\exp[-t\cdot(t_c)^{-1}]$.

ously, H_r decreased to 0.44 ± 0.07 mW·g^{-1} (n = 9) below that for CPG (t test compared with 0: t = 10.66; $p < 0.0001$; Fig. 4C). To reduce the mitocondrial Ca^{2+} uptake via uniporter (Santo-Domingo et al. 2007) 5 µmol·L^{-1} KB-R7943 was added in the presence of Tpg. This procedure induced an additional increase of +5.26 ± 1.10 mm Hg in rLVP, with a rate of raising of 0.366 ± 0.078 mm Hg·min^{-1} (n = 9). The slopes of rLVP compared with time, for each one of the 3 treatments, using one-way ANOVA for repeated pairing values from 9 experiments, showed a highly significant difference (F = 11.64; p = 0.0008). The a posteriori Tukey test indicated that only the slope of the last treatment (addition of KB-R7943) was different from the other two: CPG = CPG+Tpg < CPG+Tpg+KB-R7943 ($p < 0.001$ compared with slope of CPG, and $p < 0.01$ compared with slope of CPG+Tpg). Simultaneously, after addition of KB-R7943 to CPG+Tpg, the H_r was maintained at –0.57 ± 0.10 mW·g^{-1} below the H_r for CPG (ΔH_r was not significantly different from that induced by Tpg; $p < 0.01$ compared with 0, Fig. 4C).

Mechanisms of Ca influx during the cardioplegic treatment

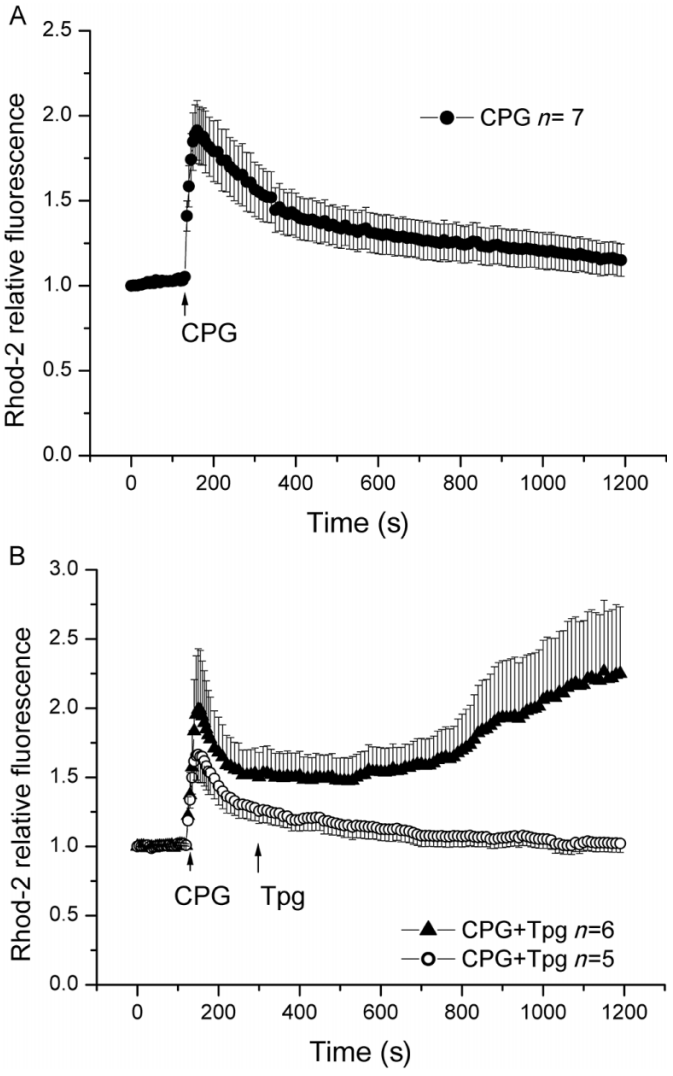
The hearts beating at 1 Hz developed a maximal LVP of 112.9 ± 9.9 mm Hg, and a H_t of 12.14 ± 1.58 mW·g^{-1} (n = 5). After cessation of electrical stimulation, the resting heat flux (H_r) was 5.07 ± 0.8 mW·g^{-1}. Figure 5A shows that when CPG was added to the hearts in the resting condition, H_r increased by 1.37 ± 0.38 mW·g^{-1} at 0.5 mmol·L^{-1} Ca, and by 2.07 ± 1.18 mW·g^{-1} at 1 mmol·L^{-1} Ca, with statistically non-significant increments in rLVP (+1.66 ± 1.12 and +2.18 ± 1.48 mm Hg, respectively, for 0.5 and 1 mmol·L^{-1} Ca). The return to Krebs-C perfusion completely reversed the effect, bringing H_r back to 4.76 ± 0.72 mW·g^{-1}, while maximum LVP during heart beat was recovered to 106.2 ± 8.5 mm Hg, and H_t was partially recovered to 8.0 ± 1.1 mW·g^{-1}, owing to the brief period of stimulation that was applied. The second addition of CPG with 1 mmol·L^{-1} Ca, reduced H_r to 5.31 ± 0.63 mW·g^{-1}. The reduction regarding the first treatment of CPG could be attributed to the reported decrease in H_r over long measurement durations (Loiselle 1987). The next addition of Nif (Ca-blocker) to CPG at 10 µmol·L^{-1} reduced H_r by 2.59 ± 0.41 mW·g^{-1} ($p < 0.05$) after 5 min without changes in LVP. Nevertheless, H_r spontaneously recovered, even while the heart was exposed to Nif (Fig. 5A).

On the other hand, blockade of the Na^+-channels by TTX did not change the resting LVP (5.08 ± 1.43 versus 5.53 ± 1.33 mm Hg) nor the H_r of hearts (before = 4.35 ± 0.10 mW·g^{-1} compared with after = 4.37 ± 0.11 mW·g^{-1} addition of TTX) under control conditions, but it reduced the initial increase in H_r evoked by CPG+1 mmol·L^{-1} Ca^{2+} (Fig. 5B). The paired differences between the ΔH_r before and after addition of TTX were significantly lower than 0 ($p < 0.05$, see the insert in Fig. 5B).

Effect of pyruvate on resting heat rate and cardiac performance

When 10 mmol·L^{-1} Pyr was added to cardioplegic hearts (25 mmol·L^{-1} K^++0.5 mmol·L^{-1} Ca^{2+}) rLVP increased slightly (2.25 ± 0.45 mm Hg, n = 20) (Fig. 6A); however, H_r increased significantly from 6.05 ± 0.13 to 9.10 ± 0.24 mW·g^{-1} (ΔH_r = +3.30 ± 0.24 mW·g^{-1}, n = 20) (Fig. 6B). To investigate whether this high energetic response was secondary to an increase in mitochondrial Ca^{2+} uptake, Pyr was added in the presence of 5 µmol·L^{-1} KB-R7943 in a subset of experiments. This resulted in a more pronounced increase in H_r regarding the hearts treated with CPG+Pyr (up to 11.04 ± 0.52 mW·g^{-1} versus 9.10 ± 0.24 mW·g^{-1}, n = 13 compared with 20, $p < 0.05$) (Fig. 6B) with a slow increase in rLVP (8.38 ± 2.05 mm Hg, Fig. 6A). When KB-R7943 was added alone to CPG, it did not significantly change H_r (from 5.90 ± 0.24 to 5.60 ± 0.26 mW·g^{-1}, n = 9, NS, Fig. 6B), but it did increase rLVP (9.90 ± 1.16 mm Hg, n = 9) to the same extent as CPG+Pyr (Fig. 6A).

Fig. 2. Changes in the mitochondrial Ca^{2+} signal estimated by the relative Rhod-2 fluorescence in rat cardiomyocytes over time, induced by CPG (25 mmol·L^{-1} K^+ + 1 mmol·L^{-1} Ca^{2+}, arrow) in a group of cells (CPG, n = 7, in Fig. 2A) and by CPG followed by addition of 5 μmol·L^{-1} thapsigargin (CPG+Tpg, n = 11, in B). Note that in B, the cells showed 2 different types of behavior (open circles and filled triangles) after the addition of Tpg. Results are expressed as mean ± SEM.

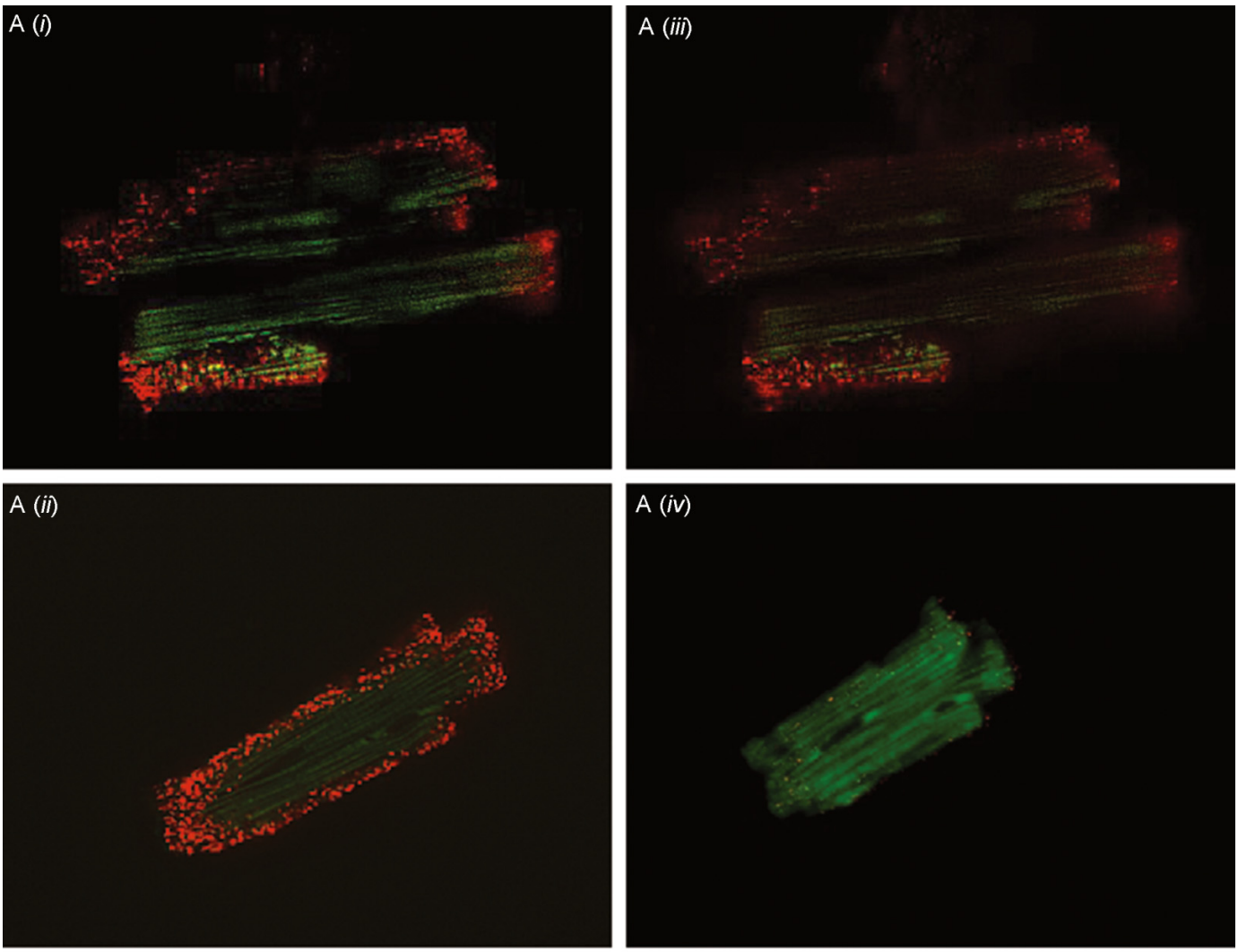


Discussion

The results show that high-K^+cardioplegia induces an increase in both $[Ca^{2+}]c$ and $[Ca^{2+}]m$ without concomitant changes in $\Delta\Psi m$. These effects were associated with a decrease in H_t of beating hearts, owing to the arrest and subsequent increase in rLVP for compared with the prior left ventricular end diastolic pressure of the beating hearts. As was previously described (Sperelakis and Schneider 1976), myocardial cells depolarize to about –45 mV in the presence of external 25 mmol·L^{-1} K^+. Consequently Ca^{2+} influx by Ca^{2+}-channels could be activated, as was demonstrated in previous studies in which verapamil addition induced a decrease in resting heat rate (H_r) under 25 mmol·L^{-1} K^+ (Holroyd et al. 1990; Márquez et al. 1997). Under this resting condition, H_r is dependent on $[Ca^{2+}]o$, since it mostly involves the mitochondrial metabolism and removal of cytosolic Ca^{2+} (Márquez et al. 1997). Although the verapamil effect could also be explained by blockade of the mNCX (Cox and Matlib 1993), it was demonstrated that a selective inhibitor of this transporter, such as clonazepam, did not reduce H_r under CPG (Ragone and Consolini 2009). Figure 5A shows that the selective and competitive Ca-blocker Nif also reduced H_r obtained during perfusion with high-K^+ low-Ca^{2+}CPG (25 mmol·L^{-1} K^+ – 1 mmol·L^{-1} Ca^{2+}). Thus, the effect of verapamil and Nif on H_r during the resting state, as well as the Ca-dependence of H_r suggest that CPG would trigger the Ca^{2+} influx by L-channels before inactivating them. On the other hand, the possibility of Ca^{2+} influx by the SL-NCX in its reverse mode, cannot be excluded (Sheu et al. 1986). When $E_{Na/Ca}$ (3 E_{Na}–2 E_{Ca}) was calculated (by assuming $[Ca^{2+}]o$ 1 mmol·L^{-1}, $[Ca^{2+}]i$ 0.1 μmol·L^{-1}, $[Na^+]o$ 130 mmol·L^{-1}, and $[Na^+]i$ 12.7 mmol·L^{-1} as initial resting conditions for the rat cardiomyocyte) (Bers 2001) a value of –58.2 mV is obtained. This value is lower than the E_m of –45 mV reported under external 25 mmol·L^{-1} K^+ (Sperelakis and Schneider 1976). Thus, it suggests that the Ca^{2+} entrance through the SL-NCX could be, at least initially, thermodynamically favored. This way of Ca^{2+} influx would explain the recovery of H_r after 5 min of Nif presence. In addition, TTX reduced the CPG-induced increase in H_r (Fig. 5B), suggesting that the slow depolarization may also activate before inactivating a fraction of Na^+-channels, causing a rise in $[Na^+]i$, which favours Ca^{2+} influx by the SL-NCX. The effect of CPG was reversible, as contractility of hearts recovered. When CPG was added during the resting state, H_r increased in 2 phases (Fig. 4A). This biphasic response was previously characterized as a transient caffeine-sensitive peak followed by a caffeine-insensitive steady state (Ponce-Hornos et al. 1992). Furthermore, the increase in H_r corresponds to the activation of the Na^+-, K^+-ATPase (measured by ^{86}Rb uptake-efflux experiments), which represents, the 95% of the steady-state and the 36% of the initial peak, respectively, (Ponce-Hornos et al. 1992). According to these studies, the exposure of the heart to CPG (high-K^+ and low-Ca^{2+} solution) results in Ca^{2+} influx by channels and reverse SL-NCX, which may trigger an initial Ca^{2+}-induced Ca^{2+}-release from the sarcoplasmic reticulum. The sarcoplasmic reticulum would remain partially depleted at low-$[Ca^{2+}]o$, but can be replenished when $[Ca^{2+}]o$ is raised under high-$[K^+]o$ (Consolini et al. 1997). Thus, it is possible that under CPG, the sarcoplasmic reticulum acts either as a store or a sink of Ca^{2+} for the interaction with sarcolemmal and mitochondrial transporters.

In the present experiments, the signals of Fura-2 (cytosolic Ca^{2+}) and Rhod-2 (mitochondrial Ca^{2+}) in cardiomyocytes were transiently increased after addition of CPG, and then exponentially decayed until a new steady-state was achieved, restoring the pattern of H_r in the whole hearts (Figs. 1, 2A, and 4A). The effect of CPG on isolated cardiomyocytes agrees with the early report in which the increase in $[Ca^{2+}]i$ was $[K^+]o$-dependent in suspended rat cardiomyocytes (Powell et al. 1984). It was also showed that the Quin-2 signal increased with low-$[Na^+]o$ + high-$[K^+]o$ in isolated cardiomyocytes (Sheu et al. 1986), and that Ca^{2+}-channels, SL-NCX, and the sarcoplasmic reticulum play a role on that response (Rathi et al. 2004). On the other hand, the increase in

Fig. 3. Estimation of changes in mitochondrial membrane potential measured as relative JC-1 fluorescence ratio (red:green) in rat cardiomyocytes. Note that the fluorescence in the centre of the cells (green, Web version only) denotes the $\Delta\Psi m$ dissipation as well as in the cell exposed to dinitrophenol (DNP), which was shortened by rigor. (A) Shows a confocal image of JC-1 fluorescence in 2 cells (*i*) before and (*ii*) after treatment with cardioplegia (CPG), and at the bottom, a confocal image of a cell (*iii*) before and (*iv*) after 5 min of treatment with 200 μmol·L^{-1} DNP. (B) shows the JC-1 fluorescence ratio (mean ± SEM) over time, before and after treatment with CPG (25 mmol·L^{-1} K^+ + 1 mmol·L^{-1} Ca^{2+}, arrow), with an unique square-point at 300 s that corresponds to the red:green ratio in a cell treated with DNP.



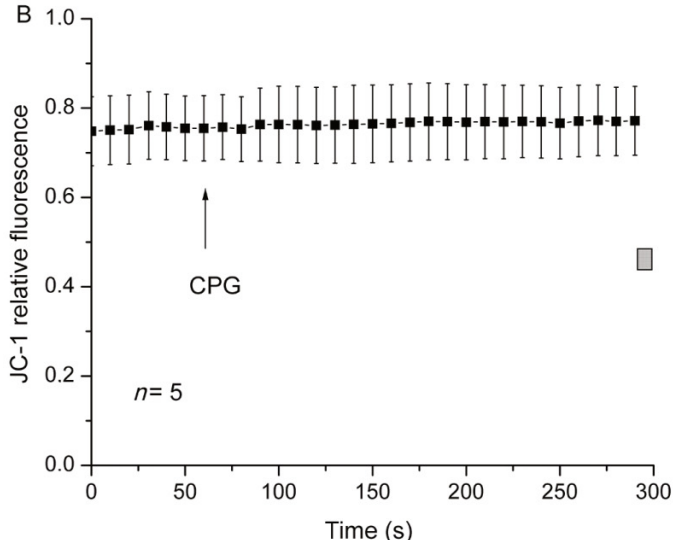
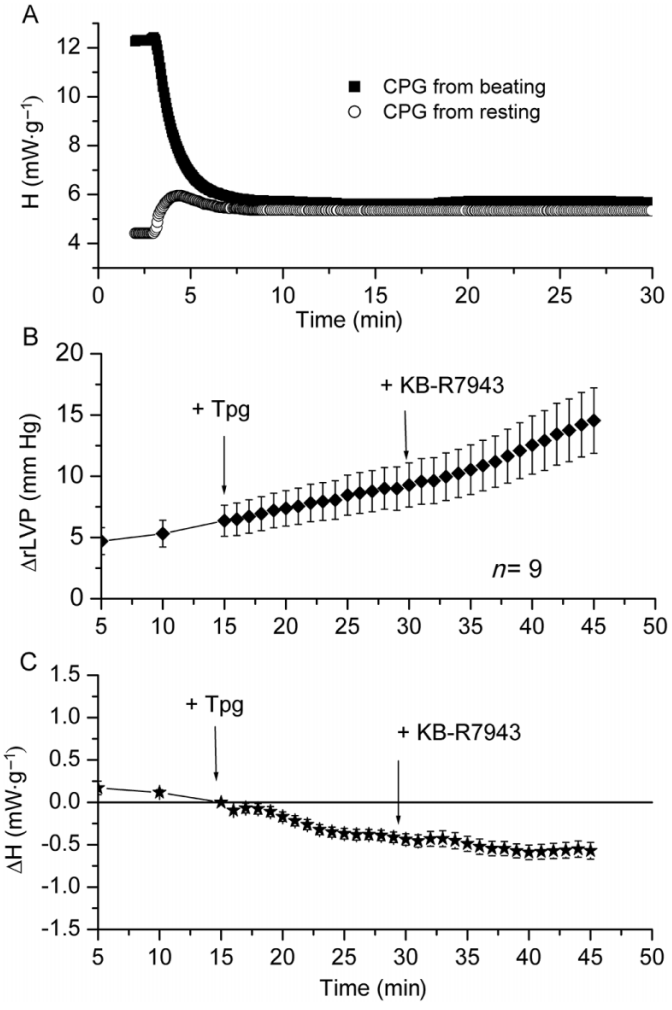


Fig. 4. (A) Typical recording of the changes in heat rate of both a beating heart (filled squares) and a resting heart (open circles), when cardioplegia (CPG, 25 mmol·L^{-1} K^+ + 1 mmol·L^{-1} Ca^{2+}) is added at the third minute. (B) Changes induced in the resting pressure (ΔrLVP, in mm Hg regarding diastolic pressure of beating hearts before CPG) of whole hearts by addition of thapsigargin (Tpg) and KB-R7943. (C) Changes induced in the resting heat rate (ΔH_r, in mW·g^{-1} regarding the value after 15 min in CPG) of whole hearts. In (B) and (C), CPG was perfused from time 0, the hearts were progressively subjected to 1 μmol·L^{-1} Tpg at 15 min (first arrow) and 5 μmol·L^{-1} KB-R7943 in the presence of Tpg at 30 min (second arrow). Results of ANOVA tests for comparing values at 15, 30, and 45 min are as follows: (B) $F = 21.06$, $p < 0.0001$; a Tukey test indicated a significant difference at $p < 0.05$ between all values. (C) $F = 37.10$; $p < 0.0001$; Tukey's test of ΔH_r at 15 compared with 30 min, $p < 0.001$; Tukey's test of ΔH_r at 30 compared with 45 min, not significant. Note in (B), the different rate of increase in ΔrLVP induced by KB-R7943 (slope, 0.366 ± 0.08 mm Hg·min^{-1}) compared with that for Tpg (slope, 0.199 ± 0.053 mm Hg·min^{-1}, $p < 0.01$, see also section on the Inhibition of SERCA and mitochondrial Ca^{2+} uniporter in cardioplegic hearts).



the Rhod-2 fluorescence caused by cardioplegia suggests that the [Ca^{2+}]m may be increased. In this context, the selectivity with which Rhod-2 detects mitochondrial Ca^{2+} has to be considered (Griffiths 2009). It has been shown that intracellular Rhod-2 could be distributed among both mitochondria and cytosol, depending on the incubation conditions. It would remain in mitochondria when cold-loading at 4 °C is followed by warming to 37 °C, favouring the hydrolysis of acetomethoxy (AM) from the dye in the cytosol and its leaching out of the cell (Trollinger et al. 1997). Other protocols that are used to avoid cytosolic distribution, such as Mn^{2+}-quenching and saponin permeabilization, or inhibition of the mitochondrial signal using the uniporter-blocker ruthenium-red, are invasive and affect mitochondrial function (O'Rourke and Blatter 2009). To avoid this, we loaded Rhod-2-AM at room temperature (about 25 °C during 1 h), as reported by Ruiz-Meana et al. (2007); however, we cannot therefore exclude the possibility of contaminant cytosolic Ca^{2+} in the signal. Nevertheless, the Rhod-2 signal decay was slower than that of Fura-2 (see Tables 1 and 2), and the changes in fluorescence after exposure to Tpg were different for both dyes. Thus, the changes in Rhod-2 signal show a Ca^{2+} compartmentalization that is different from that detected by the changes in Fura-2, and then the Rhod-2 signal must mainly correspond to mitochondrial Ca^{2+}. The differences in the decay kinetics of Rhod-2 and Fura-2 signals (Tables 1 and 2) agree with the fact that Ca^{2+} removal from mitochondria may be slower than Ca^{2+} removal from the cytosol. The exponential decay of Rhod-2 signal (time constant t_c of about 245 s) agrees with the kinetics reported by Langer (1997) in Ca^{45}-washing experiments of resting cells under high-perfusion (non-flux-limited), in which mitochondrial Ca^{2+} exponentially exchanges with a $t_{1/2}$ of 3.6 min with the extracellular space ($t_c = t_{1/2}\cdot(\ln 2)^{-1}$ = 5.2 min or 312 s). The decay of Fura-2 signal (t_c about 110 s) may be mostly attributable to SL-NCX, SL-Ca^{2+}-pump, and SERCA. According to Langer (1997), the exponential decay of Ca^{2+} in the absence of heart beats had a $t_{1/2}$ of 4 s for sarcolemma (t_c = 5.8 s) and 20–24 s for the SR leak in rat hearts. The longer decay obtained with Fura-2 suggests that cytosolic Ca^{2+} may be strongly buffered and (or) cycling with a slower compartment, such as mitochondria. Accordingly, the rise in Rhod-2 signal after blocking SERCA suggests that mitochondria take up part of the remaining Ca^{2+}, at least in a subpopulation of the cells (Fig. 2B). It is well accepted that Ca^{2+} movement in a subpopulation of mitochondria is dependent on Ca^{2+} microdomains established with the sarcoplasmic reticulum (Kuznetsov et al. 2006). Functional heterogeneity between subpopulations of mitochondria has been previously reported (Kuznetsov et al. 2006; Rutter 2006). In the present study, the JC-1 fluorescence disclosed two populations of mitochondria with different basal oxidation states and membrane potential. JC-1 has the unique property of forming red fluorescent aggregates locally and spontaneously under high mitochondrial ΔΨm, whereas the less polarized monomeric form fluoresces green (Salvioli et al. 1997). Figure 3 (Web version only) shows the JC-1 fluorescence ratio from both populations of mitochondria: those which are more polarized display a red subsarcolemma; and those which are less polarized display a green interior, as was described by others (Das et al. 2005). The JC-1 ratio fluorescence was not modified by CPG, suggesting that it does not change the mitochondrial ΔΨm. Contrarily, when the uncoupling agent DNP (which promotes H^+-uptake by the mitochondria) was added as a positive control, the JC-1 fluorescence turned mostly to green while the cell suffered rigor (see Fig. 3, Web version only). These results suggest that under CPG the mitochondria must increase metabolism, de-

Fig. 5. Changes in resting heat (H_r) of whole rat hearts perfused using the previously described protocols (see Materials and methods). (A) Values of H_r during evaluation of the effects of steady stimulation in Krebs-C (ss), resting state in Krebs-C (rest), perfusion of cardioplegia (CPG, 25 mmol·L^{-1} K^{2+}) at 0.5 and 1 mmol·L^{-1}Ca^{2+}), reversion to Krebs-C (rest and ss), and the effects of 10 μmol·L^{-1} nifedipine on CPG+ 1 mmol·L^{-1} Ca^{2+}. (B) Values of H_r during evaluation of the resting state in Krebs-C followed by exposure to CPG (25 mmol·L^{-1} K^{2+} and 1 mmol·L^{-1} Ca^{2+}) and reversion to Krebs-C (filled squares), and a second treatment with CPG after addition of 2 μmol·L^{-1} tetrodotoxin (TTX) (open circles). The inset in (B) shows the paired differences in ΔH_r produced by TTX between the second and the first treatments (filled triangles).

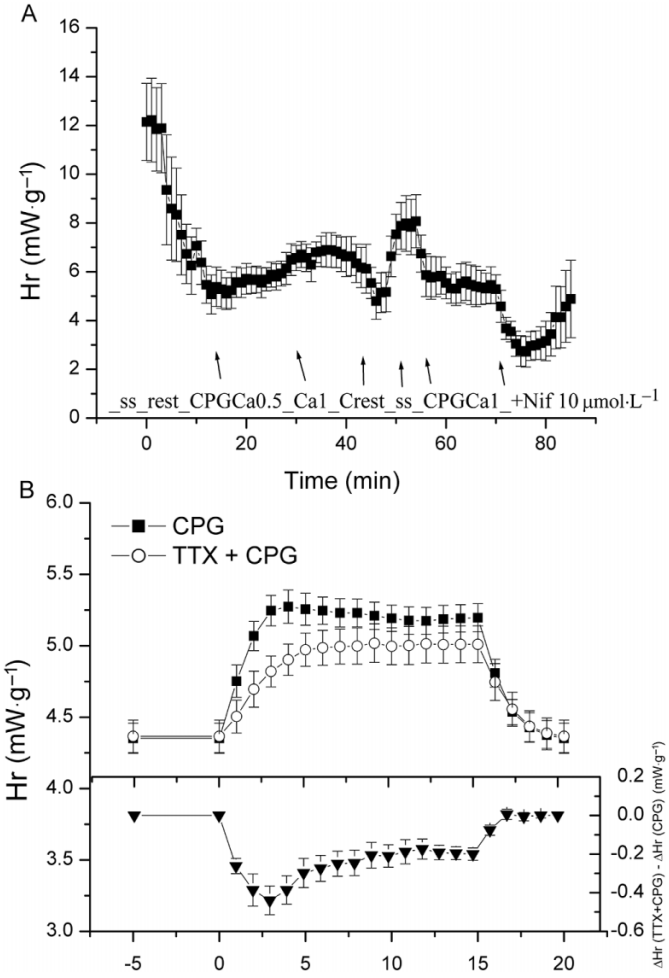
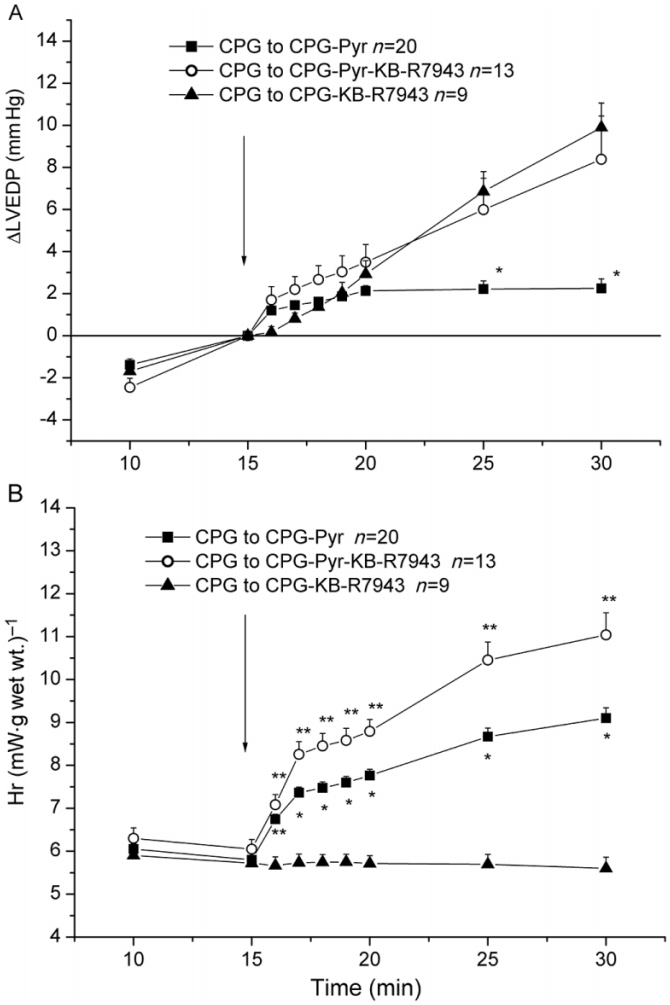


Fig. 6. Mechano-energetic performance of whole rat hearts perfused with cardioplegia (CPG, 25 mmol·L^{-1} K^+ + 0.5 mmol·L^{-1} Ca^{2+}) for up to 15 min (point taken as a reference for ΔrLVP), to which were added, in 3 groups of experiments, (*i*) 10 μmol·L^{-1} pyruvate (CPG to CPG+Pyr), (*ii*) 5 μmol·L^{-1} KB-R7943 (CPG to CPG+KB-R7943), or (*iii*) both (CPG to CPG+Pyr+KB-R7943), respectively. (A) Changes in diastolic pressure (ΔrLVP, in mm Hg). (B) Absolute resting heat rate [H_r, in mW·(g wet wt.)$^{-1}$) Two-way ANOVA for ΔrLVP in (A): by time, $F = 18.02$, $p < 0.0001$; by treatment, $F = 47.62$ $p < 0.0001$ (tests a posteriori: *, $p < 0.05$ compared with the others). (B) H_r by time, $F = 42.88$, $p < 0.0001$; H_r by treatment, $F = 223.2$, $p < 0.0001$ (tests a posteriori: asterisk (*) and double asterisk (**) show values that differ among themselves and from CPG+KB-R7943, $p \leq 0.05$). The arrow indicates the change from CPG to the respective intervention.



pending on [Ca^{2+}]m to keep the $\Delta\Psi$m constant. This is evidenced in whole hearts by the increase in H_r with CPG, with a dependence on [Ca^{2+}]o (Fig. 5), as was previously described (Márquez et al. 1997).

The pharmacological inhibition of the SR Ca^{2+} uptake by Tpg after applying CPG resulted in a further increase in Rhod-2 fluorescence in about the half of the myocytes, but always without changes in the decay of the Fura-2 signal. This suggests that the Ca^{2+} fraction not taken up by SERCA does not remain in cytosol, but rather in the mitochondria. Thus, under high-[K^+] and low-[Ca^{2+}]o, the sarcoplasmic reticulum had a low store but kept the Ca^{2+} cycling, which was interrupted by SERCA inhibition. Simultaneously, the mitochondrial Ca^{2+} uptake was increased, suggesting that both the sarcoplasmic reticulum and mitochondria complemented each other to regulate homeostasis of the cytosolic Ca^{2+}. In the whole-heart experiments, Tpg induced a decrease in H_r, suggesting that the inhibition of exothermic activity of SERCA is the main process for heat generation, despite the expected increase in mitochondrial metabolism induced by the increase in [Ca^{2+}]m. The fact that not all myocytes increased the Rhod-2 signal after SERCA inhibition

could be due to mitochondrial heterogeneity. Ca^{2+} transfer from the sarcoplasmic reticulum to mitochondria has been demonstrated in reperfused cardiac myocytes under simulated ischemia (Ruiz-Meana et al. 2009). However, Ca^{2+} movement between the sarcoplasmic reticulum and mitochondria may be bidirectional, as was detected in endothelial and HeLa cells (Griffiths 2009). Our present results suggests that Ca^{2+} normally taken up by the sarcoplasmic reticulum could be taken up by groups of mitochondria situated very near to SERCA, so that Ca^{2+} does not increase in the cytosol. Alternatively, these results suggest that under CPG there is a cycling from mitochondria to the sarcoplasmic reticulum, which was interrupted by inhibition of the SERCA. We have previously shown this in a model of stunned rat hearts exposed to CPG and ischemia-reperfusion, i.e., that inhibition of mitochondrial Ca^{2+} efflux (mNCX) by clonazepam reduced contractile recovery as well as caffeine-sensitive contracture (Consolini et al. 2007). Also, it was reported that clonazepam increased $[Ca^{2+}]m$ but reduced the cytosolic Ca^{2+} transients in beating cardiomyocytes from rats (Bell et al. 2006). Although these authors suggested that mitochondria could be contributing to the sarcoplasmic reticulum refilling (Bell et al. 2006), they did not obtain a reduction in the caffeine-sensitive SR Ca^{2+} release by clonazepam, as we found on reperfused hearts (Consolini et al. 2007). In the present experiments, the increase in Rhod-2 fluorescence without changes in Fura-2 signal after SERCA blockade under CPG, suggests a direct transfer between the two organelles when SERCA is functional, without a net diffusional Ca^{2+} movement through the cytosol.

Energetically, Tpg induced a net decrease in H_r of isolated hearts, despite the increase in rLVP, which was raised at the same rate as CPG. Then, the function of SERCA in resting cells could be replaced by other removal mechanisms. The subsequent addition of 5 $\mu mol \cdot L^{-1}$ KB-R7943 further enhanced the rate of increase of rLVP without changing H_r, which suggests an increase in Ca^{2+} binding to myofilaments. This effect could not be explained by the known inhibition of the reverse mode of the SL-NCX by KB-R7943 (Iwamoto et al. 1996). Instead, it could be due to the inhibition of the mitochondrial Ca^{2+} uniporter by KB-R7943 (Santo-Domingo et al. 2007). Thus, the simultaneous blockade of SERCA and uniporter leaves more Ca^{2+} available for actomyosin interaction. The net changes in H_r of hearts after addition of Tpg and Tpg+KB-R7943 (–0.44 and –0.57 $mW \cdot g^{-1}$, respectively) suggest an energy saving attributed to SERCA inhibition. This energy saving must exceed the exothermic events, such as the crossbridge interaction and the Ca^{2+}-uptake by mitochondria, SL-NCX, and Ca^{2+}-pumps. The fall in H_r was lower than the energy reduction expected for the SERCA inhibition, which may be in the range of –0.69 and –3.4 $mW \cdot g^{-1}$. This range was calculated by considering a SERCA stoichiometry of 2 Ca^{2+}/ATP, the equivalence of 80 kJ per mol Ca^{2+}, and the SERCA flux in the range of 17.5 and 85 nmol $Ca^{2+} \cdot g^{-1} \cdot sec^{-1}$, estimated from 42 and 207 μmol $Ca^{2+} \cdot cytosol^{-1} . s^{-1}$, respectively, for the resting state and the maximal flux measured with caffeine (Bers 2001). The consequently expected fall in heat release must be attenuated by the exothermic processes, and their contributions can be calculated as follows. The myofilament crossbridge would release 1.5 to 2.8 μW (for the change in rLVP of +2.9 to +5.3 mm Hg obtained,after Tpg and Tpg+KB-R7943, respectively, and considering the isometric energetic equivalent of 3.6 $mJ \cdot mN^{-1} \cdot mm^{-2}$ estimated by Mulieri and Alpert 1982). The cytosolic Ca^{2+} removal from mitochondria would release about 1.27 $mW \cdot g^{-1}$ under resting conditions (from the flux of 32 nmol $Ca^{2+} \cdot g^{-1} \cdot sec^{-1}$, as was described by Ponce-Hornos 1990). The sarcolemmal Ca^{2+}-pump energy can be calculated in 0.3 $mW \cdot g^{-1}$ (from the described flux of 3.7 nmol $Ca^{2+} \cdot g^{-1} \cdot sec^{-1}$), and the heat released by the SL-NCX may be about 0.16–0.32 $mW \cdot g^{-1}$ (Ponce-Hornos 1990). Then, the net change in H_r (–0.44 $mW \cdot g^{-1}$) shows that SERCA has the most important role in Ca^{2+} homeostasis. The participation of the Ca^{2+}-efflux transporters was evidenced in myocytes by the slow decrease in Fura-2 signal after the increase induced by CPG still after SERCA inhibition. Comparing the effects of SERCA blockade in both cardiomyocytes and hearts, it is seen as a contribution of Ca^{2+} to myofilaments (ΔrLVP) and to mitochondria (Rhod-2 signal) without increasing free cytosolic Ca^{2+} (Fura-2 signal). Although SERCA would be the main determinant of the energetic process, when SERCA and uniporter were simultaneously blocked by Tpg and KB-R7943, SL-NCX and the Ca^{2+}-pump likely contribute to remove cytosolic Ca^{2+} to reach the new steady state.

On the other hand, the addition of 10 $mmol \cdot L^{-1}$ Pyr to CPG increased rLVP and H_r in isolated resting hearts. Our results agree with the findings of Zima et al. (2003), who observed an increase in diastolic Ca^{2+} in intact cardiomyocytes, which was attributed to concomitant acidification of the cytosol (Gambassi et al. 1993). These authors also showed that 10 $mmol \cdot L^{-1}$ Pyr increased the amplitude of Ca^{2+} transients and SR content, which could help to explain the positive inotropism previously described in guinea pigs (Bünger et al. 1989; Tejero-Taldo et al. 1998). The Pyr-induced increments in both inotropism and Ca^{2+} transients were sensitive to the inhibition of mitochondrial Pyr uptake in rat cardiomyocytes (Zima et al. 2003; Martin et al. 1998). To investigate whether the effect of Pyr on H_r was due to an increase in mitochondrial Ca^{2+} uptake in cardioplegic hearts, the Ca^{2+} uniporter was blocked with 5 $\mu mol \cdot L^{-1}$ KB-R7943. Fig. 6 shows that addition of this drug to CPG resulted in increased rLVP, suggesting that the mitochondrial Ca^{2+} uniporter contributes to removing the cytosolic Ca^{2+} under CPG. Considering the previously discussed increase in Rhod-2 signal in cardiomyocytes after SERCA blockade, then once again the results suggest that both mechanisms complement each other in the removal of cytosolic Ca^{2+}. Nevertheless, the addition of KB-R7943 to CPG did not change H_r, which suggests that the energetic cost for the increase in rLVP (crossbridge and cytosolic Ca^{2+} removal) was compensated by the inhibition of Ca^{2+} uptake in the mitochondria. Nevertheless, KB-R7943 did not reduce the effect of Pyr on rLVP or H_r, suggesting that in the resting state, Pyr does not stimulate the mitochondrial Ca^{2+}-uptake. Instead, KB-R7943 increased the effect of 10 $mmol \cdot L^{-1}$ Pyr on both H_r and rLVP. Comparing the 3 treatments shown in Fig. 6, the increase in rLVP could be mostly attributed to KB-R7943, but H_r was increased by Pyr still more when the $[Ca^{2+}]m$ was increased by blocking the uniporter. Thus, it can be concluded that Pyr increases a basal Ca^{2+}-independent mitochondrial activity in the resting state under CPG.

In summary, the results of this work demonstrate that high-K^+ cardioplegia increases cytosolic and mitochondrial Ca^{2+} by activating Ca^{2+} influx via L-type Ca $^{2+}$ channels and the reverse mode of SL-NCX. Also, there is a Ca^{2+} transfer between mitochondria and the sarcoplasmic reticulum, even during the resting state. A reduction in SERCA activity may contribute to both a mitochondrial Ca^{2+} overload and the reduction of the energetic cost of the heart, although sarcolemmal mechanisms contribute to a slow Ca^{2+} loss. Inhibition of the mitochondrial uniporter may also induce diastolic contracture with the consequent energy expenditure. Thus, these mechanisms could be important in pathological conditions such as ischemia-reperfusion injury, in which SR function is compromised. Our results could also give support to the hypothesis that there is a Ca^{2+} movement between mitochondria and the sarcoplasmic reticulum, which could contribute to the organelle's refilling when SERCA is reactivated during reperfusion. Moreover, addition of Pyr during CPG may increase the resting cardiac energy expenditure, mostly by increasing the mitochondrial metabolism, which could explain, at least in part, its cardioprotective properties.

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