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ARTICLE

Effects of pyruvate on the energetics of rat ventricles stunned by ischemia–reperfusion

Patricia Bonazzola, María Inés Ragone, and Alicia E. Consolini

Abstract: Pyruvate (Pyr) was proposed as an additive to cold high-K^+–low-Ca^{2+} cardioplegia (CPG) to protect the heart during surgery. We explored whether Pyr and CPG would work synergistically to protect rat hearts from stunning during ischemia–reperfusion (I/R). Using confocal microscopy with Fluo-4 and Rhod-2, respectively, we measured the heat release and contractility of perfused ventricles during I/R, and the cytosolic and mitochondrial [Ca^{2+}] in cardiomyocytes. We found that under cold-CPG (30 °C), 10 mmol·L^{-1} Pyr reduced the post-ischemic contractile recovery (PICR) as well as muscle economy, when added either before I or during I/R, which was reversed by blockade of UCam. In noncardioplegic hearts, Pyr was cardioprotective when it was present during I/R, more so at 37 °C than at 30 °C, with improved economy. In cardiomyocytes, the addition of Pyr to CPG slightly increased the mitochondrial [Ca^{2+}] but decreased cytosolic [Ca^{2+}]. The results suggest that Pyr only protects hearts from stunning when present before ischemia and during reperfusion, and that it dampens the cardioprotective properties of CPG. The mechanisms underlying such different behavior depend on the dynamic balance between Pyr stimulation of the energetic state and mitochondrial Ca^{2+} uptake. Our results support the use of Pyr in stunned hearts, but not in cold high-K^+ cardioplegia.

Key words: pyruvate, heart, ischemia–reperfusion, cardioplegia, calorimetry, Ca^{2+}.

Résumé : Le pyruvate (Pyr) a été proposé comme additif lors d'une cardioplégie (CPG) froide avec K^+ élevé et Ca^{+2} faible, afin de protéger le cœur lors de la chirurgie. Les auteurs ont exploré si le Pyr protégeait les cœurs de rats de la sidération myocardique durant une ischémie–reperfusion (I/R) de manière synergique à la CPG. Ils ont mesuré la contractilité et la libération de chaleur de ventricules perfusés durant l'I/R, ainsi que la [Ca^{2+}] cytosolique et mitochondriale des cardiomyocytes par microscopie confocale en présence de Fluo-4 et Rhod-2, respectivement. Les auteurs ont trouvé qu'en condition de CPG froide (30 °C), une concentration de 10 mmol·L^{-1} de Pyr réduisait la récupération contractile post-ischémique (RCPI) ainsi que l'économie musculaire, lorsqu'il était ajouté avant l'I ou durant l'I/R, ce qui était renversé par un blocage UCam. Chez les cœurs non cardioplégiques, le Pyr exerçait une activité cardioprotectrice lorsqu'il était présent durant l'I/R, davantage à 37 °C qu'à 30 °C, avec une économie améliorée. Chez les cardiomyocytes, l'ajout de Pyr à la CPG accroissait légèrement la [Ca^{2+}] mitochondriale mais diminuait celle du cytosol. Les résultats suggèrent que le Pyr protège uniquement les cœurs de la sidération myocardique lorsqu'il est présent avant l'ischémie et durant la reperfusion, mais qu'il atténue la cardioprotection conférée par la CPG. Les mécanismes sous-jacents à de tels comportements dépendent de la balance dynamique entre la stimulation par le Pyr de l'état énergétique et la captation de Ca^{2+} mitochondrial. Ces résultats appuient l'utilisation de Pyr chez les cœurs en sidération myocardique, mais non lors d'une cardioplégie froide avec K^+ élevé. [Traduit par la Rédaction]

Mots-clés : pyruvate, cœur, ischémie–reperfusion, cardioplégie, calorimétrie, Ca^{2+}.

Introduction

The consequences of ischemia and reperfusion in the heart depend on the degree of injury. Although cardiac infarction is the most studied condition, it is still important to find new therapeutic strategies to prevent the impacts of brief ischemic episodes. Such events lead to a heart with depressed contractility once the perfusion has been reestablished, a state known as "stunning" (Braunwald and Kloner 1982). Several positive inotropic interventions, including pyruvate (Pyr) (Mentzer et al. 1989; Mallet et al. 2005; Keweloh et al. 2007; Schillinger et al. 2011), have been proposed to improve this condition without increasing the heart rate. Injection of Pyr in the coronary artery of dogs (1 millilitre of a 150 mmol·L^{-1} solution per minute) before and after ischemia improved the contractile function, but only during the administration (Mentzer et al. 1989). More recently, Pyr has been used to increase cardiac performance in patients with chronic and acute heart failure, resulting in beneficial effects for their cardiac index (Schillinger et al. 2011). Thus, it was proposed as an alternative to catecolamines (Mallet et al. 2005; Keweloh et al. 2007). Pyr at

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Abbreviations: ΔLVP, area under the curve of ΔLVP in caffeine contractures; C, control perfusion; caff–low-Na, Krebs-C with 10 mmol·L^{-1} caffeine and 36 mmol·L^{-1} Na; CPG, high-K^+–low-Ca^{2+} crystalloid cardioplegia; DMSO, dimethylsulfoxide; H_r, resting heat rate; H_t, total heat rate; I, non-flow ischemia; I/R, ischemia–reperfusion; KBR, KB-R7943; LVP, left ventricular pressure; ΔLVP, changes in left ventricular pressure; ΔLVEDP, changes in left ventricular end diastolic pressure; m-NCX, mitochondrial Na–Ca exchanger; NHE, sarcolemmal Na–H exchanger; *P*, maximal pressure development in contraction; d*P*/d*t*, first derivative of *P*; P/H_t, total muscle economy; PICR, post-ischemic contractile recovery; Pyr, pyruvate; R, reperfusion; RyR channel, ryanodine receptor channel; SERCA, sarcoreticular calcium pump; SL-NCX, sarcolemmal Na–Ca exchanger; SR, sarcoplasmic reticulum; TCA, tricarboxilic acid cycle; *t*PP, time to peak pressure of contraction; *t*R, time of relaxation; UCam, mitochondrial Ca^{2+} uniporter.

P. Bonazzola. Cátedra de Biofísica, Facultad de Odontología e Instituto de Investigaciones Cardiológicas (CONICET, Facultad de Medicina), Universidad de Buenos Aires (UBA), Argentina.
M.I. Ragone and A.E. Consolini. Cátedra de Farmacología, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), 47 y 115 (1900) La Plata, Argentina.
Corresponding author: Alicia E. Consolini (e-mail: dinamia@biol.unlp.edu.ar).

supraphysiological concentrations (2–10 mmol·L^{-1}) also improved cardiac contractility in isolated and intact hearts (Bünger et al. 1989; Martin et al. 1998; Mallet and Sun 1999). The inotropic properties of Pyr, which entail complex mechanisms, are thought to be caused by an increase in cytosolic phosphorylation potential (Zweier and Jacobus 1987; Bünger et al. 1989). A better cardiac energetic state would lead to better Ca^{2+} handling and higher release by the sarcoplasmic reticulum (SR). In support of this theory is the loss of inotropic effects whenever the mitochondrial uptake of Pyr is impaired (Martin et al. 1998; Zima et al. 2003). Another contribution of Pyr to inotropism is increased myofilament sensitivity to Ca^{2+} (Torres et al. 2013). This could be counteracted by direct inhibition of the RyR channel and steady intracellular acidification (Zima et al. 2003; Kockskämper et al. 2005). At the metabolic level, Pyr is an aerobic substrate that induces acidosis. This is caused by the cellular uptake of H^+ and Pyr by a symporter; a process similar to the mitochondrial pyruvate carrier (MPC) used to enter to mitochondria (Halestrap and Price 1999). Furthermore, Pyr becomes a fuel for mitochondrial oxidative metabolism when added to exogenous glucose (Damico et al. 1996). On the other hand, Pyr has antioxidant properties (De Boer et al. 1993; Bassenge et al. 2000; Mallet and Sun 2003) owing to its chemical structure and anaplerotic mitochondrial metabolism. These properties ultimately lead to a rise in the cytosolic NADPH necessary to maintain antioxidant cell defenses (Mallet and Sun 2003). Thus, the reduction in infarct size has been attributed to the antioxidant properties of Pyr (Kristo et al. 2004).

Since Pyr induces positive inotropism without tachycardia, it has been proposed as an additive to cardioplegia in cardiac surgeries, to prevent stunning and mitigate the oxidative stress and damage (Knott et al. 2005, 2006; Ryou et al. 2010). Cardioplegic solutions, such as the cold high-$[K^+]$ crystalloids (Dobson 2004), are used in some surgical procedures to arrest the heart. It is well known that these solutions are protective against ischemia–reperfusion (I/R) dysfunction. This protection is effected by reducing the cardiac total energy consumption (Stowe et al. 2000; Wang et al. 2003). In previous works, we studied the energetics of a model of beating hearts (rat) perfused with cold cardioplegia (high-K^+–low-Ca^{2+} crystalloid at 30 °C) before subjecting them to I/R. It prevented diastolic contracture and improved the post-ischemic contractile recovery and the muscle economy (Consolini et al. 2004, 2007). Now we want to evaluate possible cardioprotective synergism between Pyr with the cold cardioplegic solution. Although there are several reports about the effect of Pyr on metabolism (Bünger et al. 1989; Mallet and Sun 2003), only a few show its effects on muscle heat release (Chapman and Gibbs 1974; Daut and Elzinga 1989), and they were done in aerobic papillary or trabecular muscles, none in ventricles under I/R. Recently we have shown that Pyr increases the basal heat flux associated with metabolism when added to cardioplegia in perfused rat ventricles (Consolini et al. 2011). The aim of this work was to evaluate whether Pyr and CPG are additive as cardioprotectives in the stunned heart, and the energetic consequences on post-ischemic hearts. Online calorimetry was employed, since it was extensively used for studying the energetics of Ca^{2+} movements in resting and beating hearts (Ponce-Hornos et al. 1982, 1995; Bonazzola and Takara 2010), as well as under ischemia and hypoperfusion (~~Ponce-Hornos et al. 1982, 1995,~~ Consolini et al. 2007; Ragone et al. 2013). The myothermic analysis in ventricles was complemented with measurements of mitochondrial and cytosolic $[Ca^{2+}]$ in ventricular cardiomyocytes to suggest the underlying mechanisms of Pyr, either added to a cold cardioplegia or as a unique pretreatment in a model of cardiac stunning.

Materials and methods

Animals

We used adult Wistar–Kyoto rats (200–280 g, both sexes). Animals were bred either at the animal facilities of the ININCA in the School of Medicine, UBA–CONICET, or the School of Veterinary Sciences at UNLP. Animals were housed at room temperature with a 12 h (light) – 12 (dark) cycle, with access to food and water ad-libitum. This study was approved by our local institutional ethics committee. Animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care (CCAC 1984, 1993, Vol. 1 (2nd ed., 1993) and Vol. 2 (1984).

Mechano-calorimetric measurements in perfused ventricles

Biological preparation and mechanical measurements

The animals were injected with heparin (2000 U) and given an overdose of pentobarbital (50 mg·kg^{-1}). The hearts were immediately excised and perfused using the Langendorff method with Krebs solution at a constant flow rate of 6 mL·min^{-1}·g^{-1}, using a peristaltic pump (Gilson Minipuls 3) (Ponce-Hornos et al. 1995; Consolini et al. 2001; Consolini and Bonazzola 2008; Bonazzola and Takara 2010; Ragone et al. 2013). The atria were removed, and spontaneous beating was stopped by a little cut on the interventricular septum close to the aorta. A latex balloon was introduced in the left ventricle, connected to a pressure transducer (Gould Statham P23 dB) with a flexible cannula. While continuously perfused, the heart was introduced into the chamber of a calorimeter (Ponce-Hornos et al. 1982), which was closed and submerged in a bath kept at either 30 or 37 °C, depending on the protocol. The perfused ventricles were stimulated by 2 electrodes inside the chamber. Square pulses of 5 V per 5 ms were generated at 1 Hz (at 30 °C) or at 3 Hz (at 37 °C) with an electrical stimulator (Grass model SD9, Braintree, Massachusetts, USA). The experimental protocol (working temperature, heart rate, and duration of I/R) were selected based on previous studies (Consolini and Bonazzola 2008; Ragone and Consolini 2009) to evaluate stunning in conditions nearer to cold cardioplegia (30 °C and 1 Hz). When a more physiological condition at 37 °C was used, a heart rate of 3 Hz was chosen because in spite of this being low for the rat, ~~because~~ at that rate coronary flow was not compromised and kept the heart demand balanced with the perfusion flow.

Left ventricular pressure (LVP) at optimal volume and total heat rate (H_t) were continuously and simultaneously recorded as described below. LVP was recorded either with a Grass ~~M~~model 7 polygraph (Braintree, Mass.) connected to an A/D converter (Axon Instruments ~~Inc.~~, Foster City, California, USA), or with a digital acquisition system, PowerLab 2/26, 2 channels (ADInstruments). Maximal pressure development during the isovolumetric contraction (P), and changes in diastolic pressure with respect to the preischemic control perfusion (ΔLVEDP) were calculated during ischemia and reperfusion. The maximal contraction and relaxation rates were obtained from the first derivative (dP/dt), from which the contraction times were measured. Thus, the time to peak-pressure (tPP) and the relaxation time (tR) were calculated, respectively, as the period between the start of contraction and the zero-derivative or maximal pressure development (tPP), and the period between the peak-pressure and the end of the contraction (tR). The period between the start and the end of the contraction was the total-time ($tt = t\text{PP} + t\text{R}$). Contractility was expressed as absolute P (mm Hg; 1 mm = 133.322 Pa) or as a percentage of the pre-ischemic steady value of P, under control perfusion for each heart.

Calorimetric measurements

Our flux calorimeter was built from a large mass of copper with an internal chamber, containing 2 insulated ceramic thermoelectric modules (Melchor Thermoelectrics) with a total of 254 thermosensitive junctions (Ponce-Hornos et al. 1982; 1995). The

minimum output of the thermosensitive units of the calorimeter was higher than 10 μV, whereas the electrical noise was about 1 μV at maximum gain (1 μV·mm^{-1}). The calorimeter was submerged in a water bath at either 30 °C or 37 °C, while the temperature was controlled with a heating–cooling (±0.03 °C) triple bath, where the perfusion solution in one of the baths was also thermally equilibrated. So, the units detected temperature differences between the internal chamber with the heart and the external bath regulated at constant temperature. After the heat and pressure stabilization of the hearts, the heat rate (H_t) was continuously measured in the presence or absence of perfusion (I/R period). This process was also done without muscle to estimate base lines before and after each experiment. At the end of each experiment, we performed a calorimetric calibration by passing a known electrical power (2 mW, 2.1 kHz, <1 V), which is subthreshold for the heart, inside the calorimetric chamber either under perfusion and no-flow conditions. Heat output was recorded at the same time as LVP in the respective acquisition device. Results are expressed either as H_t in milliWatts per gram of wet weight or as a percentage of the pre-ischemic steady value for H_t under control conditions for each heart. Total muscle economy (P/H_t) was calculated from the measured P and H_t.

Protocols

All perfusion protocols were initiated as follows: the perfused ventricles were stabilized for 45 min. To avoid changes in osmolarity with the solution containing 10 mmol·L^{-1} Pyr, the Krebs perfusion was replaced with another Krebs solution containing 20 mmol·L^{-1} sucrose (control perfusion, C) prior to initiating the experimental protocol. This new steady state for the beating hearts was considered the "initial control value" of P and H_t. The ventricles were then exposed to different pretreatments, as shown in Fig. 1. Perfusion was maintained for either 15 or 30 min before ischemia. In most protocols, the experiment was conducted at 30 °C and the heart was stimulated at 1 Hz. In one protocol the experiments were performed at 37 °C and 3 Hz, as indicated in Fig. 1. Pretreatments were followed by 45 min of global nonflow ischemia and 45 min reperfusion with a Krebs solution supplemented with either sucrose or 10 mmol·L^{-1} Pyr (R+Pyr) (see Fig. 1).

Before ischemia, hearts were pretreated as follows:

(*i*) Effects of Pyr with cardioplegia: to evaluate the effect of 10 mmol·L^{-1} Pyr on cardioplegic cardioprotection, ventricles were perfused with a mixture of Krebs – 25 mmol·L^{-1} K^+ – 0.5 mmol·L^{-1} Ca^{2+} – 20 mmol.L^{-1} sucrose (CPG) before ischemia at 30 °C in the following sequence: (1) C–CPG–I/R; (2) C–CPG–CPG+Pyr–I/R; (3) C–CPG–CPG+Pyr–I/R+Pyr (Fig. 1).

To evaluate whether the effect of 10 mmol·L^{-1} Pyr was associated with the activation of the uniporter (UCam), the protocol was (4) C–CPG–CPG+Pyr+KBR–I/R (where KBR is KB-R7943 at 5 μmol·L^{-1}) (Fig. 1).

(*ii*) Effects of Pyr without cardioplegia: to evaluate the effect of 10 mmol·L^{-1} Pyr before, during, and after ischemia in non-cardioplegic ventricles at 30 °C and 1 Hz, the treatments were as follows: (5) C–I/R; (6) C–C+Pyr–I/R; (7) C–C+Pyr–I/R+Pyr; (8) C–I/R+Pyr (Fig. 1).

To evaluate whether the effect of Pyr was concentration-dependent, a group of ventricles was pretreated with a lower concentration, 3 mmol·L^{-1} Pyr before ischemia, as in protocol 9 (Fig. 1) C–C+3 mmol·L^{-1} Pyr–I/R.

To evaluate whether the effect of 10 mmol·L^{-1} Pyr depended on the cardiac demand, hearts were exposed to I/R under more challenging conditions (37 °C and 3 Hz of heart rate) in 2 groups: (10) $(C–I/R)_{37}$ and (11) $(C–C+Pyr–I/R+Pyr)_{37}$ (Fig. 1). To obtain a stunning model with about the same post-ischemic contractile recovery (PICR) as those ventricles in the model at 30 °C, these hearts were exposed to 20 min of ischemia and 45 min of reperfusion.

(*iii*) Effects of Pyr with cardioplegia on the SR store: to indirectly evaluate the effect of Pyr added to CPG on the post-ischemic caffeine-sensitive SR-Ca^{2+} store, the protocols included pretreatments analogous to the basic protocols 1, 2, and 4 (Fig. 1), plus another group of ventricles pretreated with C–CPG–CPG+KBR. All of them were exposed to 45 min of ischemia and reperfused with Krebs that contained 10 mmol·L^{-1} caffeine-36 mmol·L^{-1} Na^+ to release the SR store and prevent Ca^{2+} efflux through the SL-NCX (see protocols 12, 13, 14, and 15 in Fig. 1). The changes in left ventricular pressure (ΔLVP) during I/R were measured over the control preischemic diastolic level. The area under the curve for ΔLVP vs. time during reperfusion was calculated over the ischemic value. The area under the curve of absolute values of H_t vs. time was also calculated.

Confocal microscopy in isolated cardiomyocytes

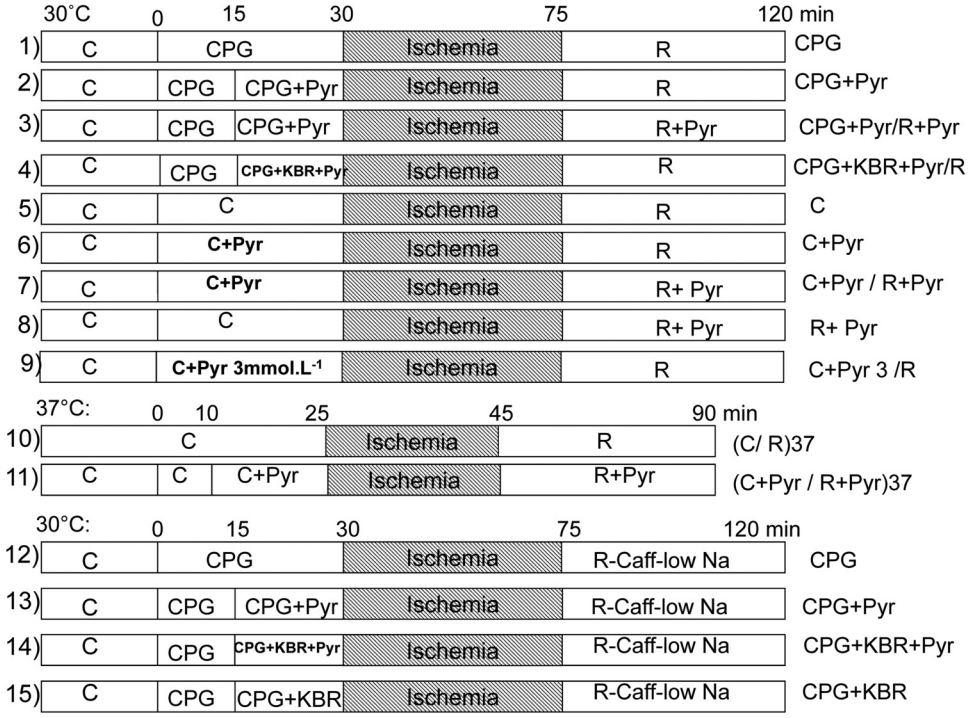
Isolation of cardiac myocytes

Ventricular myocytes were isolated from adult rats (200–280 g body mass) as previously described (Bridge et al. 1990). Once the animal was anesthetized, the heart was quickly removed and placed in a Langendorff system. The heart was perfused with a modified Krebs–24-HEPES solution virtually free of Ca^{2+} for 5 min. The solution was then changed to Krebs–24-HEPES with 50 μmol·L^{-1} $CaCl_2$, 0.1 mg·mL^{-1} collagenase P (Roche), and 0.02 mg·mL^{-1} protease XIV (Sigma–Aldrich, St. Louis, Missouri, USA). After 14 min, the solution was again changed to an enzyme free 50 μmol·L^{-1} Ca^{2+} Krebs–24-HEPES solution. All perfusion solutions were bubbled with O_2 and maintained at 37 °C. Following this procedure, the ventricles were removed and minced. The pieces were shaken in the low-Ca^{2+} solution for 10 min and then filtered. The Ca^{2+} concentration was raised in steps to 1 mmol·L^{-1} Ca^{2+} and the isolated ventricular cells were stored for up to 6 h in this HEPES-buffered saline solution.

Confocal microscopy

To measure intracellular free Ca^{2+}, isolated rat cardiomyocytes were loaded with Fluo-4 AM (12 μmol·L^{-1}; Molecular Probes–Invitrogen, Carlsbad, Calif.) for 15 min at room temperature. Whenever mitochondrial free Ca^{2+} was tested, cells were loaded with Rhod-2 AM (3 μmol·L^{-1}; Molecular Probes–Invitrogen) during 1 h at 4 °C followed by wash-up for at least 1 h at 37 °C (Trollinger et al. 1997; Ragone et al. 2013). Cells were placed on a laminin-precoated perfusion chamber and superfused with Krebs–24-HEPES solution containing 2 mmol·L^{-1} Ca^{2+} until stabilization. Then, fluorescence signals were recorded using a confocal microscope Leica SP5 (Leica Microsystems, Mannheim, Germany), as previously described (Ragone et al. 2013). Data were analyzed with the Leica LAS AF Lite version 2.2.1 software. Self-ratio emission fluorescence intensity (F/F_o) was calculated and, to avoid bleaching effects on the fluorescence signal, data obtained during the start and the end of Krebs-C perfusion were fitted by a nonlinear fit (Origin 7.0, OriginLab Corporation, Northampton, Mass.) and considered the baseline. Results are expressed as changes in the F/Fo ratio ($\Delta F/F_o$) above the fitted baseline. To evaluate cytosolic free Ca^{2+}, Fluo-4 loaded cells were excited at 488 nm every 20 s for 15 min. Changes in fluorescence emission at wavelengths higher than 505 nm from defined cell regions were monitored over time. To investigate mitochondrial free Ca^{2+}, Rhod-2 loaded cells were excited at 540 nm every 20 s for 15 to 20 min, and the changes in fluorescence emission at wavelengths higher than 560 nm from defined cell regions were monitored over time. The protocol began with the basal condition of myocytes in the chamber perfused with Krebs–24-HEPES solution (C) for 3 min, followed by 5 min of perfusion either with CPG–24-HEPES (CPG) or with Pyr 10 mmol·L^{-1} added to either CPG–24-HEPES (CPG+Pyr) or Krebs–24-HEPES (C+Pyr).

Fig. 1. Protocols in isolated perfused ventricles (described in the section on *Materials and methods*). The time of each treatment is indicated in the top of each series, considering zero time after the stabilization in control perfusion (C) and before [illegible] respective pretreatment. The reperfusion (R) [illegible] done with Krebs-C except w[illegible] indicated. C, Krebs-C; CPG, cardioplegia 25 mmol·(L [illegible])$^{-1}$ at 0.5 mmol·(L Ca^{2+})$^{-1}$; caff, 10 mmol·(L [illegible]feine)$^{-1}$; low Na, 36 mmol·(L [illegible])$^{-1}$; KBR, KB-R7943 at 5 μmol·L^{-1}; Pyr, pyruvate 10 mmol·L^{-1} except when indicated at 3 mmol·L^{-1} (Pyr-3 mmol·L^{-1}).



Solutions and drugs

The hearts were perfused with a Krebs solution containing (in mmol·L^{-1}): 118 NaCl, 7 KCl, 0.5 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$, and 6 dextrose, bubbled with 95% O_2–5% CO_2. To avoid changes in osmolarity with solutions containing pyruvate, we added sucrose (20 mmol.L^{-1}) to the Krebs solution (C). Pyruvate was added at 10 mmol·L^{-1} (C+Pyr). As in previous studies (Consolini et al. 2004, 2007), an external high potassium ($[K]_o$) – low calcium ($[Ca]_o$) solution was used as a model of crystalloid cardioplegia (CPG) by changing the respective saline concentrations of Krebs-C to the following (in mmol·L^{-1}): 25 KCl, 100 NaCl, and 0.5 $CaCl_2$, and adding 20 mmol·L^{-1} sucrose. Whenever indicated, reperfusion was done with Krebs containing 10 mmol·L^{-1} caffeine, 2 mmol·L^{-1} Ca^{2+}, and 36 mmol·L^{-1} Na^+ (caff–low-Na) (Consolini et al. 2007). The solution with low $[Na^+]_e$ was prepared by replacing 107 mmol·L^{-1} NaCl in the C solution with sucrose.

The solution for cardiomyocyte isolation contained (in mmol·L^{-1}): 126 NaCl, 4.4 KCl, 1.0 NaH_2PO_4, 5 $MgCl_2$, 24 HEPES, 22 dextrose, 20 taurine, 5 creatine, 0.5 Na-pyruvate, adjusted with NaOH to pH 7.4 and bubbled with O_2 100%. During the experiment, cardiomyocytes were superfused in Krebs–24-HEPES solution, containing (in mmol·L^{-1}): 127 NaCl, 4.4 KCl, 1 $MgCl_2$, 24 HEPES, 2 $CaCl_2$, and 11 dextrose, adjusted to pH 7.4 with NaOH and bubbled with 100% O_2. Probenecid (0.5 mmol·L^{-1}) was added for the Fluo-4 experiments. CPG in 24-HEPES was obtained from the Krebs–24-HEPES solution by changing the respective concentrations to (in mmol·L^{-1}): 106 NaCl, 25 KCl, and 1 $CaCl_2$ (CPG – 1 mmol·L^{-1} Ca^{2+}).

Pyruvic acid sodium salt (ICN Biomedicals, Aurora, Ohio, USA) was dissolved in Krebs the day of experiment or, less frequently, diluted from a 10 mol·L^{-1} aqueous stock solution prepared during the week and kept frozen at –20 °C. KB-R7943 (Tocris-Cookson, Ellisville, Miss.) was diluted in Krebs from a stock solution (12 mmol·L^{-1} in dimethylsulfoxide; DMSO). Caffeine (ICN, Costa Mesa, Calif.) was directly dissolved in Krebs.

Statistical analysis

Results are the mean ± SEM. Multiple comparisons by 2-way ANOVA for repeated measures (factors treatment and time) were done for each parameter. "A posteriori" Bonferroni paired tests were conducted among treatments, and their results are shown in each figure. Also, paired or unpaired Student *t*-tests were used whenever appropriate. Values for $p < 0.05$ were considered statistically significant. All statistical analyses were performed with Graph Pad Prism version 4.

Results

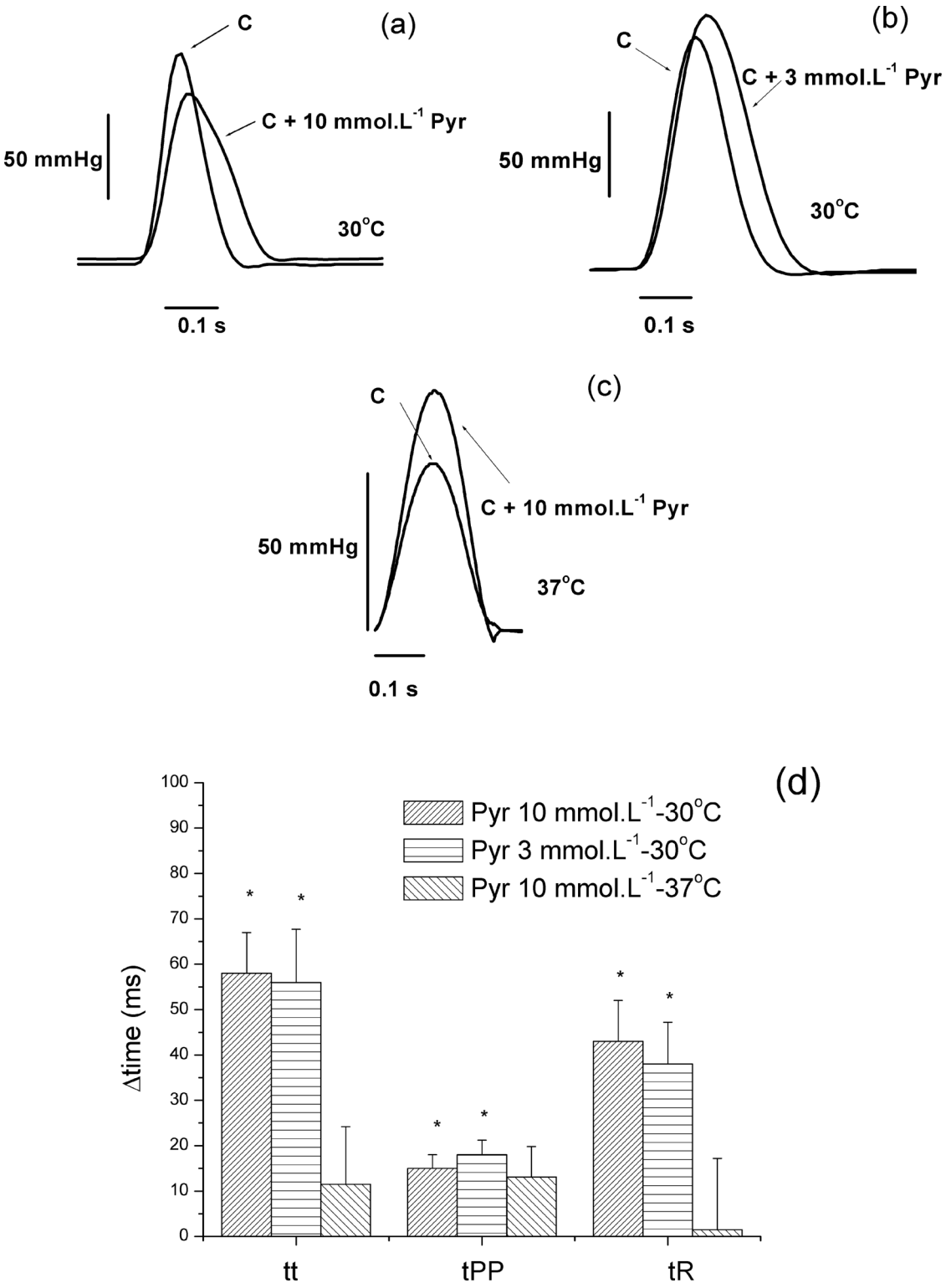
Effects of pyruvate on ventricles

Effects on pre-ischemic behavior

When the ventricles were perfused with Krebs solution at 30 °C and stimulated at 1 Hz, the addition of 10 mmol·L^{-1} Pyr induced a transient decrease in *P* that was partially recovered towards control values after 3–4 min, so that *P* was reduced from 106.8 ± 7.8 to 84.3 ± 6.9 mm Hg ($p < 0.05$; representing –20.1% ± 3.1%, $n = 24$). Pyr also induced lengthening of both the time to peak pressure of contraction (*t*PP) and that of relaxation (*t*R) (Figs. 2*a* and 2*d*). F2
Simultaneously, Pyr changed the total heat flux of beating hearts (H_t) from 11.5 ± 0.5 to 13.0 ± 0.6 mW·g^{-1} ($p < 0.05$). The total muscle economy (P/H_t) was reduced from 9.6 ± 0.7 to 6.7 ± 0.6 mm Hg·mW^{-1}·g^{-1} ($p = 0.0029$). Nevertheless, when Pyr was added at 3 mmol·L^{-1} it induced a slight positive inotropism of +7.1% ± 1.9% ($n = 5$; Fig. 2*b*) associated with an increase in H_t from 13.5 ± 1.3 to 15.9 ± 1.0 mW·g^{-1} ($p < 0.05$) without significant change in total economy (9.1 ± 1.2 vs. 8.1 ± 0.9 mm Hg·mW^{-1}·g^{-1}). Figures 2*b* and 2*d* shows that at this lower concentration, Pyr also prolonged *t*PP and *t*R.

On the other hand, when ventricles were perfused at the more physiological condition of 37 °C and stimulated at 3 Hz, 10 mmol·L^{-1} Pyr induced a positive inotropism of +85.4% ± 19.7%

Fig. 2. Changes induced by pyruvate (Pyr) on the pre-ischemic contraction of perfused ventricles. Typical superimposed records of left ventricular pressure in Krebs-C (C) and (*a*) Krebs-C with 10 mmol·L⁻¹ Pyr at 30 °C–1 Hz, (*b*) C and 3 mmol·L⁻¹ Pyr at 30 °C–1 Hz, and (*c*) C and 10 mmol·L⁻¹ Pyr at 37 °C–3 Hz. (*d*) Changes induced to the periods of contraction: total time (*t*t), time to peak-pressure (*t*PP), and time of relaxation (*t*R). (*, $p < 0.05$ vs. zero, $n = 24$, 5, and 5, for the conditions shown in (*a*), (*b*), and (*c*), respectively).

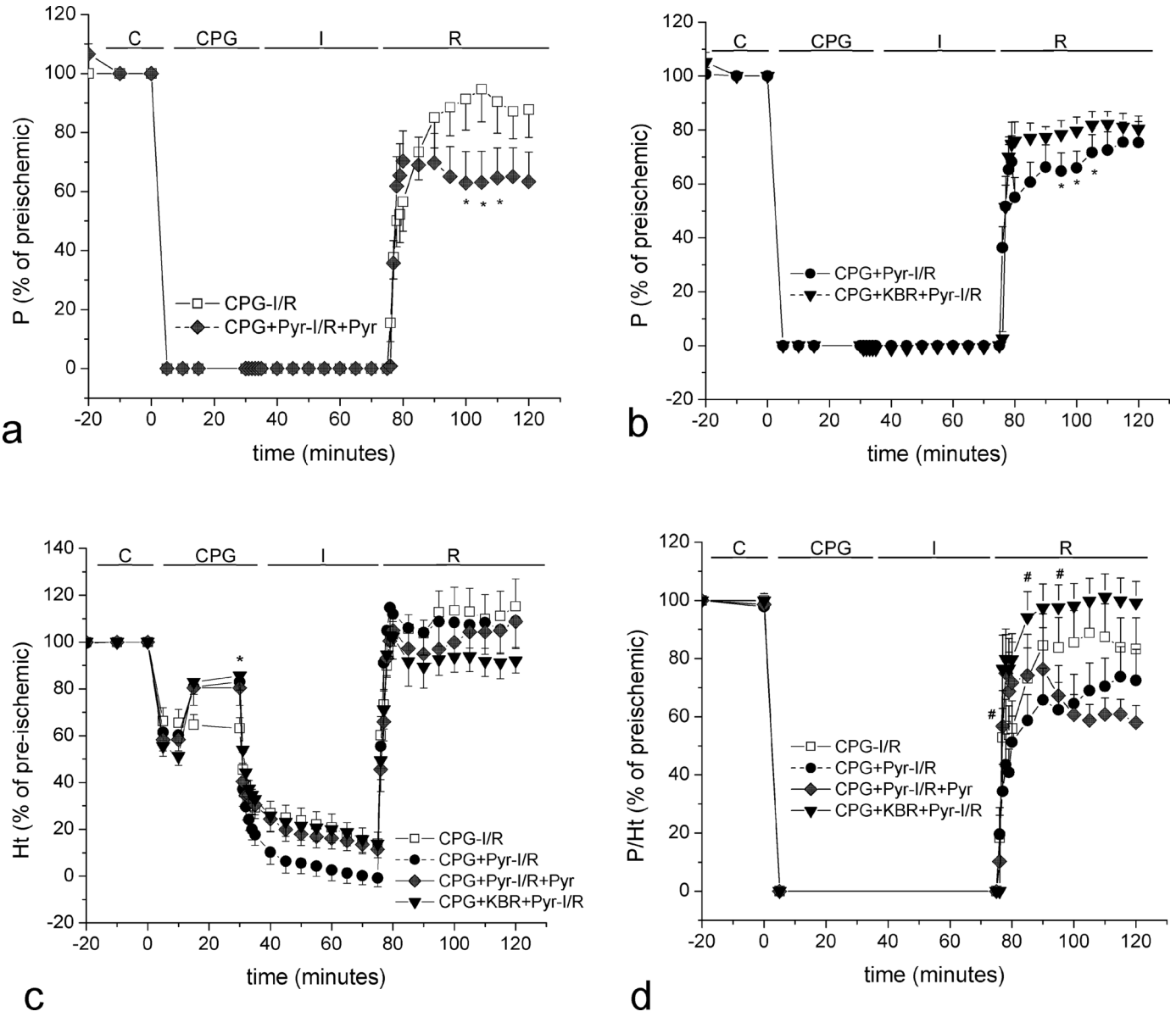


with respect to C ($n = 5$; Fig. 2*c*) without changing H_t (16.3 ± 2.4 vs, 16.6 ± 1.7 mW·g⁻¹), so that total muscle economy was increased (from 3.9 ± 0.8 to 7.5 ± 2.3 mm Hg·mW⁻¹·g⁻¹). Figures 2*c* and 2*d* show that 10 mmol·L⁻¹ Pyr did not prolonged the times of contraction and relaxation at 37 °C.

Effects of Pyr on CPG cardioprotection

When Pyr was added at 10 mmol·L⁻¹ to CPG in the model of cold cardioplegia at 30 °C, the arrested hearts significantly increased the resting heat rate (H_r) from 6.0 ± 0.1 to 9.4 ± 0.3 mW.g⁻¹ ($p < 0.05$). This pretreatment with CPG+Pyr reduced the PICR when

Fig. 3. Effects of 10 mmol·L^{-1} pyruvate (Pyr) on hearts pretreated with 25 mmol·L^{-1} K$^+$ – 0.5 mmol·L^{-1} Ca$^+$ cardioplegia at 30 °C and 1 Hz, and exposed to 45 min no-flow ischemia (I) and 45 min reperfusion with Krebs-C (R). Results correspond to the protocols shown in Fig. 1 as follows: CPG (□, n = 13); CPG+Pyr-I/R (●, n = 15); CPG+Pyr-I/R+Pyr (◆), (n = 10); and CPG+KBR+yr (▼, n = 7), where KBR is 5 μmol·L^{-1} KB-R7943. Results are the mean ± SEM of values expressed as a percentage of the initial measurement in Krebs-C for maximal pressure development (P, in 3*a* and 3*b*), heat flux rate (H_t in 3*c*), and muscle economy (P/H_t in 3*d*). Post-hoc tests: *, $p < 0.05$ when compared with CPG-I/R; #, $p < 0.05$ when compared with CPG+Pyr-I/R (see also the results for the 2-way ANOVA in Table 1).



F3 compared with CPG alone (Figs. 3*a*–3*b*), without changing the recovery of H_t (Fig. 3*c*). The total muscle economy P/H_t had a tendency to be reduced (Fig. 3*d*). As Pyr increases aerobic metabolism, favoring the driving force for the UCam, we assessed whether the reduced PICR was related to a Pyr stimulation of mitochondrial Ca^{2+} uptake during the beat. For this purpose, KB-R7943 at 5 μmol·L^{-1} (KBR) was used to block UCam. Ventricles pretreated with CPG+KBR+Pyr developed a PICR not different from hearts treated with CPG alone, thus partially reversing the negative effects of Pyr (Fig. 3*b*). To understand whether Pyr was cardioprotective during reperfusion, a group of ventricles was pretreated with CPG+Pyr at 10 mmol·L^{-1} and reperfused also in the presence of Pyr (R+Pyr). These ventricles developed a PICR not different from that of ventricles only pretreated with CPG+Pyr (Figs. 3*a*–3*b*) (2-way T1 ANOVA in Table 1). The recovery of ventricular H_t was similar to that of CPG ventricles (Fig. 3*c*). Muscle economy P/H_t was increased in hearts pretreated with CPG+KBR+Pyr when compared with those treated with CPG+Pyr (Fig. 3*d*). None of the conditions altered the changes in LVEDP during I/R (results not shown).

Effects of pyruvate during I/R in the absence of CPG

To understand whether the lack of protection of Pyr in cardioplegic ventricles exposed to I/R was due to some interaction with CPG, we evaluated the effects of 10 mmol·L^{-1} Pyr in Krebs-C at 30 °C and 1 Hz, before and during I/R. Neither the pretreatment with Pyr nor the reperfusion with Pyr significantly changed PICR. However, the presence of Pyr before ischemia and during the entire I/R significantly improved PICR at the start of reperfusion (Fig. 4*b*) with a reduction in the calorimetrical recovery (H_t%; F4 Fig. 4*c*). The beneficial effect of Pyr was also evident in the reduc-

Table 1. Results from the 2-way ANOVA applied to contractility and heat release (as % of the initial *C* value, or as absolute value) during the protocols showed respectively in Figs. 3, 4, and 6, and described in Fig. 1.

	Figure 3		Figure 4		Figure 6	
Statistics	%*P*	%H_t	%*P*	%H_t	ΔLVP (mm Hg)	H_t (mW·g^{-1})
	By treatment					
F	5.142	2.744	33.92	9.943	255.9	6.91
DF	3	3	3	3	3	3
p	0.0015	0.0419	<0.0001	<0.0001	<0.0001	0.0001
	By time					
F	179.8	96.15	259.2	171.1	188.5	53.11
DF	32	32	27	27	73	73
p <	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
	Interaction					
F	1.485	1.004	2.336	1.248	2.531	1.982
DF	96	96	81	81	219	DF = 219
p	0.0022	0.4716	<0.0001	0.0762	<0.0001	<0.0001
DF residual	1322	1322	812	812	1702	1702
		P/H_t	ΔLVEDP (mm Hg)	%P/H_t		
	By treatment					
F		23.22	27.32	80.34		
DF		3	3	3		
p <		0.0001	0.0001	0.0001		
	By time					
F		49.10	64.78	224.8		
DF		32	27	27		
p <		0.0001	0.0001	0.0001		
	Interaction					
F		1.467	2.327	4.972		
DF		96	81	81		
p		0.0232	<0.0001	<0.0001		
DF residual		799	812	812		

Note: DF, degrees of freedom. Results of "a posteriori" tests are shown in the figures.

tion of diastolic contracture (ΔLVEDP) developed during reperfusion (Fig. 4*a*). Thus, muscle economy evaluated from the P/H_t ratio was significantly improved when Pyr was present from before ischemia to throughout reperfusion (Fig. 4*d*).

To evaluate whether the negative inotropic effects of Pyr pretreatment were due to its excessive concentration, we added Pyr at 3 mmol·L^{-1} into the Krebs-C solution and perfused the ventricles at 30 °C and 1 Hz before ischemia. Under these conditions, both *P* and H_t recovered to about 90% during reperfusion (Fig. 5*a*), and the muscle economy was kept constant with values of 8.1 ± 0.9 (before ischemia) and 8.8 ± 0.7 (n = 5) mm Hg·mW^{-1}·g^{-1} (after 45 min reperfusion).

To evaluate whether the effect of Pyr was influenced by the low energetic demand, ventricles were exposed to a more physiological condition at 37 °C and 3 Hz. Under this protocol, addition of Pyr (10 mmol·L^{-1}) before, during, and after ischemia increased the PICR to 153% ± 11% of the control pre-ischemic condition (Fig. 5*b*, 2-way ANOVA compared with C: by treatment $F = 293.1$, $p < 0.0001$; and by time $F = 7$, $p < 0.0001$), while H_t returned to initial values (Fig. 5*c*). Consequently, the total economy P/H_t was increased by the presence of Pyr before I/R (4.5 ± 0.7 vs. 7.5 ± 2.3 mm Hg·mW^{-1}·g^{-1}), and remained at about 90% to the end of reperfusion (6.5 ± 1.5 mm Hg·mW^{-1}·g^{-1}).

Effects of pyruvate and CPG on sarcoreticular Ca^{2+}

We evaluated whether the Pyr reduction of PICR was due to a decrease in the SR calcium content. Analogous pretreatments were conducted in hearts exposed to CPG and ischemia, but now reperfused with a Krebs solution containing 10 mmol·L^{-1} caffeine and 36 mmol·L^{-1} Na^+ (R-caff–low-Na). With this solution, most of the SR Ca^{2+} content will be released while avoiding Ca^{2+} efflux via the SL-NCX. Under these conditions, R-caff–low-Na induced a contracture whose area under the curve (AUC-ΔLVP) was dependent on the SR Ca^{2+} content. Figures 6*a*–6*b* show that neither Pyr (10 mmol·L^{-1}) nor Pyr plus KB-R7943 (5 μmol·L^{-1}) added to the CPG pretreatment changed the caffeine-dependent contracture or the associated energetic output. Nevertheless, when KB-R7943 was added to CPG it induced a raise in the caffeine-dependent contracture over the ischemic line with an AUC-ΔLVP of 3960 ± 355 vs. 2716 ± 178 mm Hg·min^{-1} (t = 2.968, $p < 0.0128$), for CPG+KBR and CPG, respectively (Fig. 6*a*; Table 1). The energetic output associated with the caffeine-induced contracture was similar for all conditions, although Pyr increased the resting heat rate (H_r) prior to I/R (Fig. 6*b*; Table 1) as previously reported (Consolini et al. 2011).

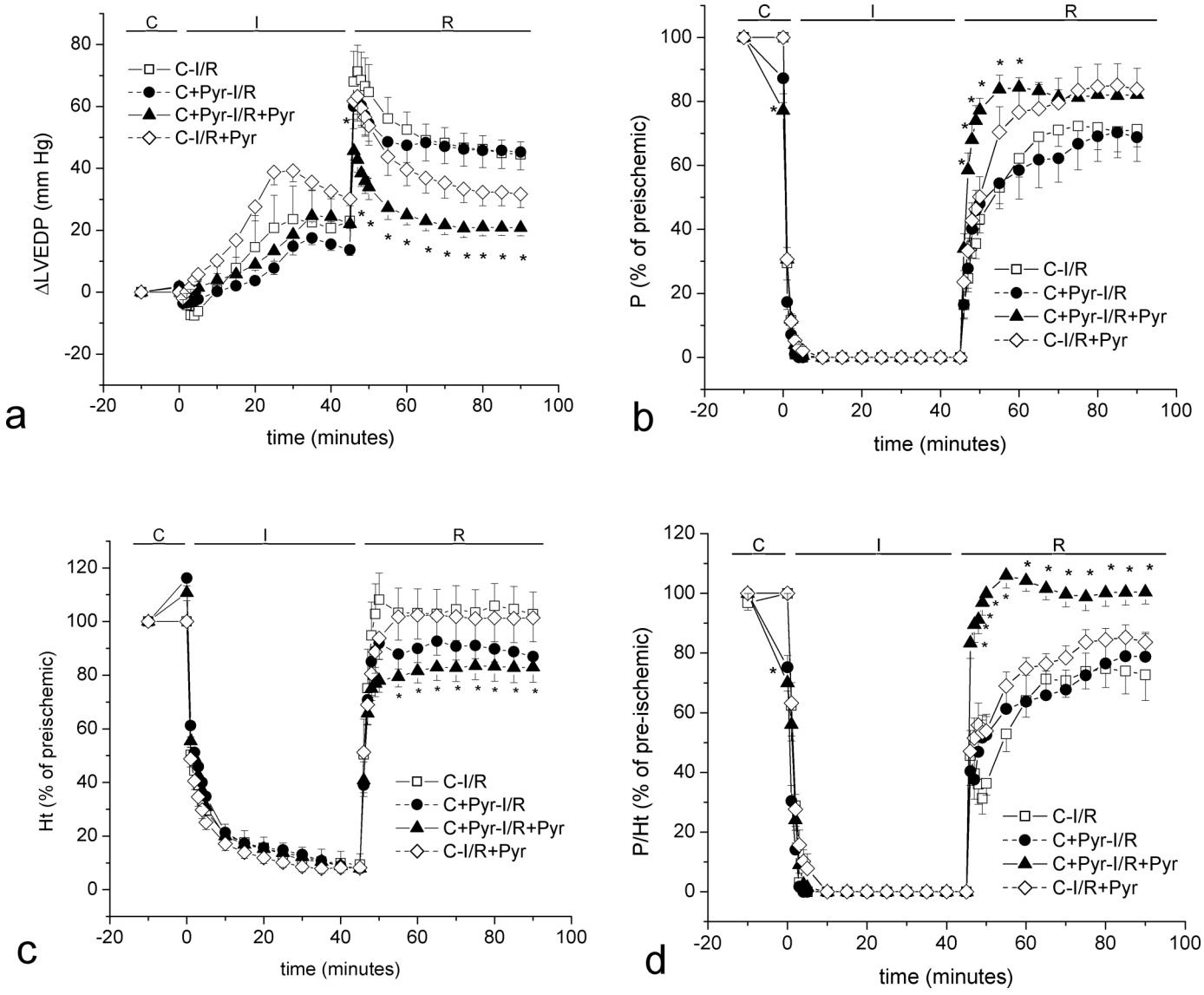
Effects of pyruvate on cytosolic and mitochondrial Ca^{2+} in cardiomyocytes

We have previously shown that adding 1 mmol·L^{-1} Ca^{2+} to CPG increased the cytosolic free Ca^{2+}, as estimated with Fura-2, in rat myocytes (Consolini et al. 2011) and with Fluo-4 in guinea-pig cardiomyocytes (Ragone et al. 2013). The addition of Pyr decreased the Fluo-4 F/F_o signal, suggesting a Ca^{2+} redistribution (Fig. 7*a*). On the other hand, the behavior for the mitochondrial Ca^{2+} estimated by the Rhod-2 signal was different to that of Fluo-4. CPG increased the Rhod-2 signal, while the addition of Pyr raised the Rhod-2 signal more in Krebs-C (Fig. 7*c*) than in CPG, in which it stayed at a steady level (Fig. 7*b*).

Discussion

This work gives new insights on the effects of Pyr on hearts stunned by I/R. Even though this metabolic substrate was used in heart failure due to the positive inotropism, this work demonstrates that it was not effective when added to cold cardioplegic solution before an ischemic insult in rat ventricles. However, the results suggested that Pyr could be useful to prevent stunning induced by I/R only when it is present before and during the reperfusion. The origin of such effects will be discussed below.

Fig. 4. Effects of 10 mmol·L^{-1} pyruvate (Pyr) on hearts exposed to 45 min no-flow ischemia (I) and 45 min reperfusion with Krebs-C (R) at 30 °C and 1 Hz. Results correspond to the protocols shown in Fig. 1 as follows: C–I/R (□, n = 8); C+Pyr–I/R (●, n = 10); C+Pyr-I/R+Pyr (▲, n = 7); and C–I/R+Pyr (◇, n = 7). Results are the mean ± SEM of values expressed as the change in left ventricular end diastolic pressure over preischemic (ΔLVEDP, in 4*a*), and as a percentage of the initial value measured in Krebs-C for maximal pressure development (P, in 4*b*), heat flux rate (H_t in 4*c*), and muscle economy (P/H_t in 4*d*). Post-hoc tests: *, $p < 0.05$ when compared with C–I/R (see also the results for the 2-way ANOVA in Table 1).



Effects of Pyr in pre-ischemic perfused ventricles

The negative inotropism of 10 mmol·L^{-1} Pyr seen in our experiments at 30 °C (Fig. 2*a*) is in accordance to the tendency found in other reports (Kerr et al. 1999; Diolez et al. 2007). However, some studies found that Pyr is a positive inotropic agent in several species, e.g., rabbit and human (Leite-Moreira et al. 2002; Keweloh et al. 2007; Torres et al. 2013), dog (Mentzer et al. 1989; Yanos et al. 1994), guinea-pig (Daut and Elzinga 1989), and rat (Martin et al. 1998), while others did not find any inotropic change in rat hearts (Ojha et al. 2012). This difference in behavior can be attributed to several factors, such as in vivo vs. in vitro experiments, temperature, and concentration. Also, the negative inotropism may be associated with the intracellular acidosis induced by Pyr, especially at concentrations as high as 10 mmol·L^{-1} (Zima et al. 2003; Kockskämper et al. 2005). In fact, when Pyr was perfused at lower concentrations (3 mmol·L^{-1}), contractility was slightly raised (Fig. 2*b*). At the more physiological temperature (37 °C), metabolism increases, thus stimulating the cellular active transport systems such as MPC and SERCA. These effects could explain the increased contractility of 10 mmol·L^{-1} Pyr at this temperature (Fig. 2*c*). The temperature-dependence of Pyr effect may be the result of a balance among its several cellular effects. At a physiological temperature, the positive inotropism of Pyr suggests the predominance of mechanisms activated by the rise in aerobic metabolism, such as phosphorylation potential, SR Ca^{2+} store, and myofilament Ca^{2+} sensitivity (Zima et al. 2003; Mallet et al. 2005; Torres et al. 2013). They would be exceeding the negative inotropic effects derived from acidosis and inhibition of RyR (Zima et al. 2003). Regarding acidosis, it is paradoxical that although the rate of sarcolemmal Na–H exchanger (NHE) rises with temperature, a change of +10 °C induced a cytosolic acidification of about 0.1 pH units due to intracellular buffers (Ch'en et al. 2003). Because of this, it is not expected that the acidosis induced by Pyr would be attenuated at 37 °C. Positive inotropism would

Fig. 5. Influence of concentration and cardiac demand on the effects of pyruvate (Pyr) on P and H_t on ventricles exposed to stunning by ischemia–reperfusion (I/R). Results correspond to the protocols shown in Fig. 1 as follows: 3 mmol·L^{-1} Pyr in Krebs-C at 30 °C and 1 Hz, and exposed to 45 min no-flow ischemia (I) and 45 min reperfusion with Krebs-C (R) (in 5*a*, $n = 5$, compare with C-I/R in Fig. 4); and 10 mmol·L^{-1} Pyr in Krebs-C at 37 °C and 3 Hz, and exposed to 20 min ischemia and 45 min of reperfusion with Krebs-C+Pyr (◇ C+Pyr–I/R+Pyr, $n = 5$) in comparison with the protocol of C–I/R at 37 °C and 3 Hz (◆, $n = 7$) (in 5*b* and 5*c*). Results are the mean ± SEM of values expressed as a percentage of the initial measurement in Krebs-C.

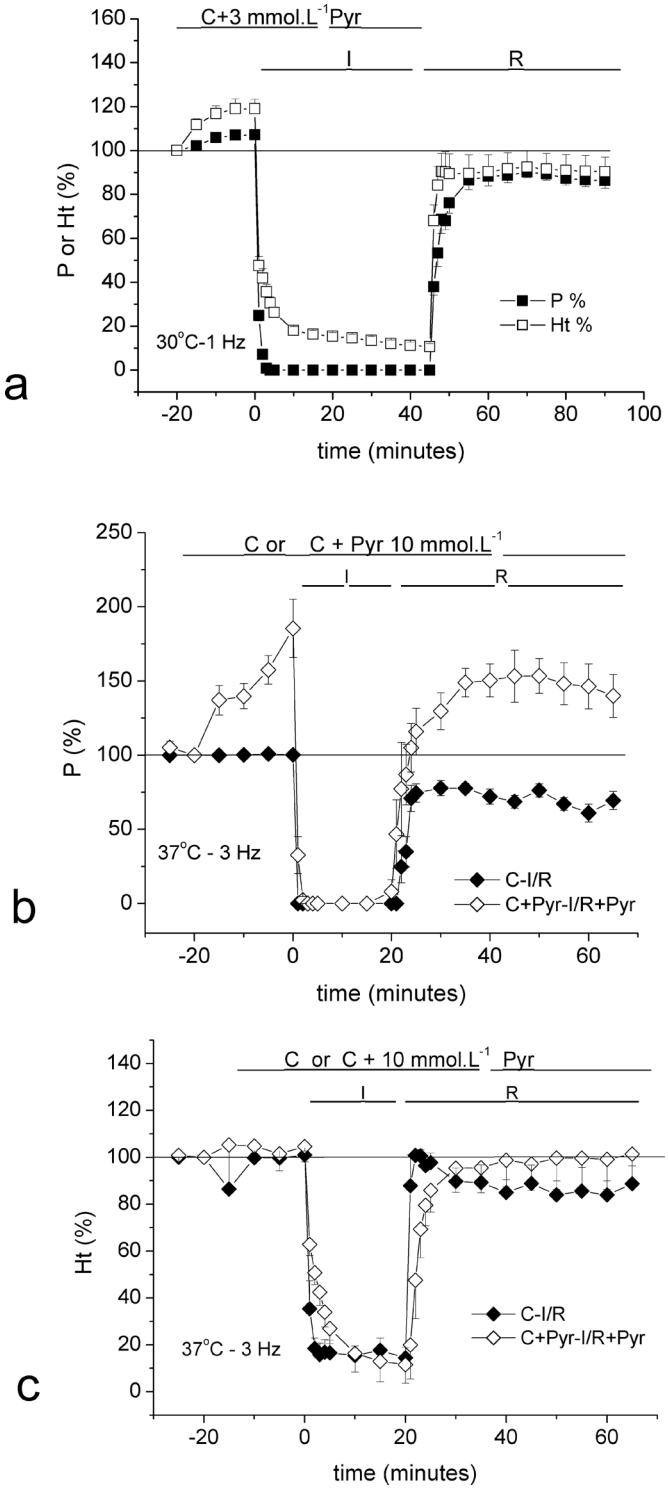
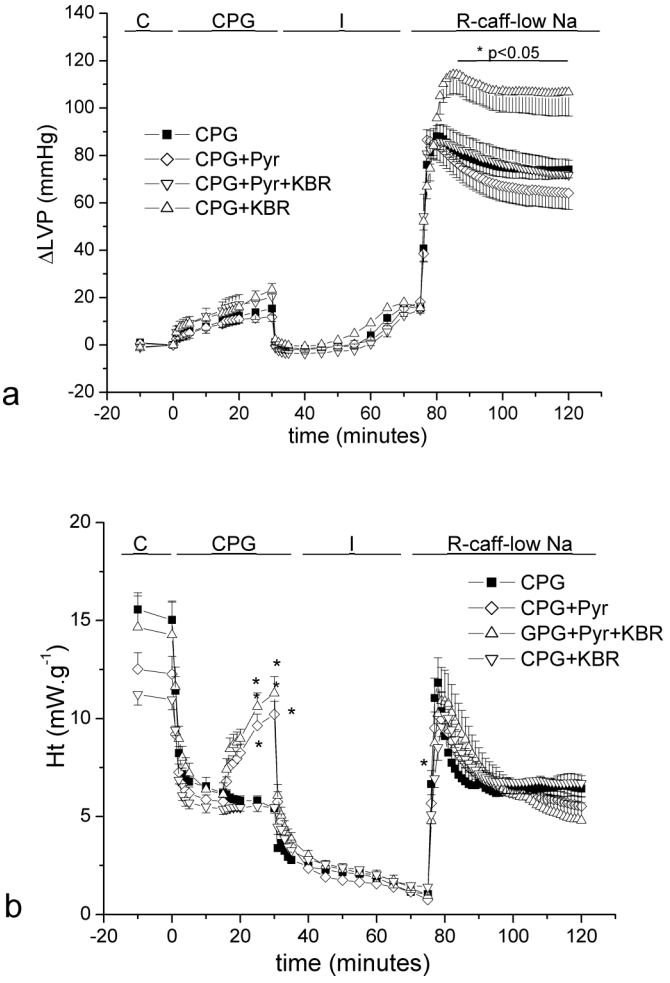
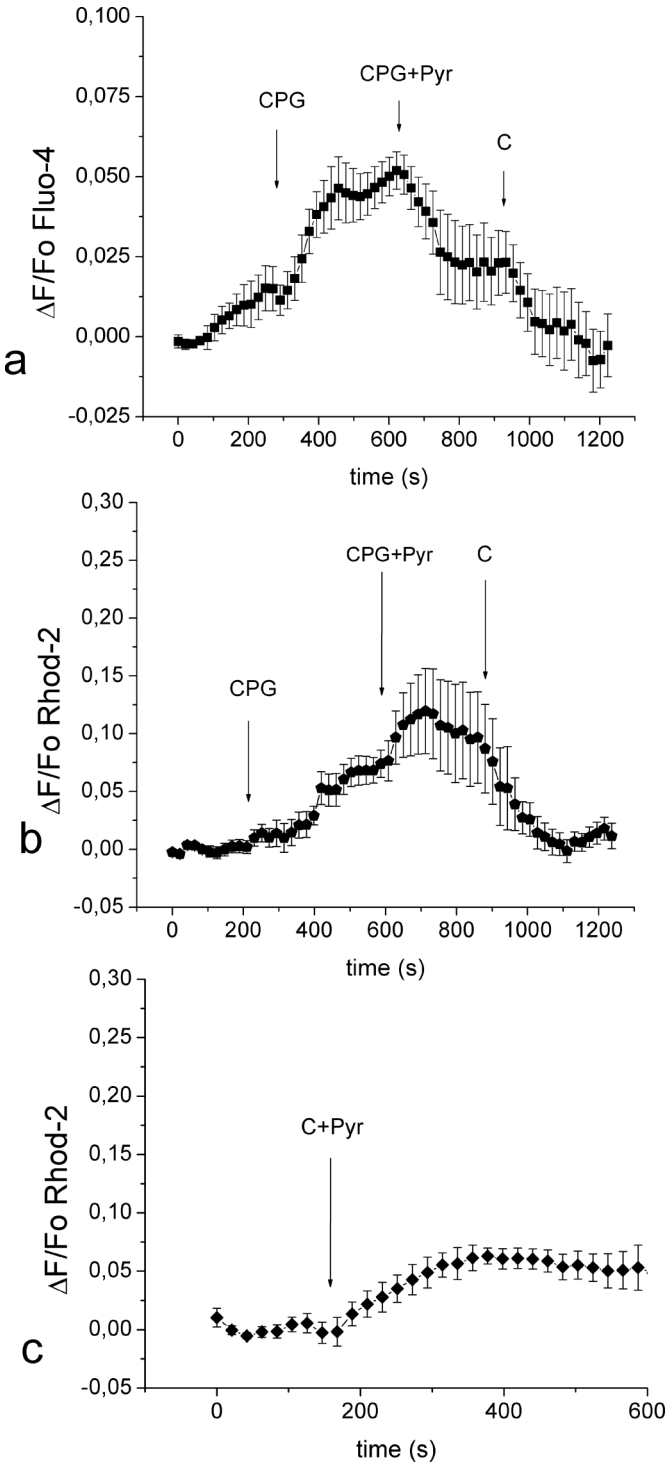


Fig. 6. Effects of 10 mmol·L^{-1} pyruvate (Pyr) on the contractures induced by reperfusion with Krebs with 10 mmol·L^{-1} caffeine, 36 mmol·L^{-1} Na^+, and 2 mmol·L^{-1} Ca^{2+} (R–caff–low-Na). Ventricles were exposed to the protocols shown in Fig. 1 as follows: CPG (□, $n = 6$); CPG+Pyr (◇, $n = 8$); CPG+KBR+Pyr (▽, $n = 6$); and CPG+KBR (△, $n = 7$), and exposed to 45 min ischemia (I) followed by 45 min reperfusion at 30 °C and 1 Hz. Results (mean ± SEM) show the changes in left ventricular pressure of arrested hearts over the initial diastolic value in C (ΔLVP, in 6*a*) and the heat rate flux (H_t, in 6*b*) (see also the results for the 2-way ANOVA in Table 1); *, $p < 0.05$ compared with the CPG group by a-posteriori tests.



depend on Pyr activation of the TCA cycle at optimal temperatures, which increases the phosphorylation potential (Hak et al. 1992). Moreover, optimal temperatures could favor the Pyr stimulation of the myofilament Ca^{2+} sensitivity (Torres et al. 2013). In mitochondria, Pyr acts as a substrate to the electron transport chain and generation of the proton-motive gradient, which is the driving force for the Ca^{2+} uptake by the UCam. In fact, in our confocal results, 10 mmol·L^{-1} Pyr increased the Rhod-2 signal of cardiomyocytes (Fig. 7*c*), suggesting an increase in free mitochondrial Ca^{2+}. The $[Ca^{2+}]m$ mainly depends on the balance between influx through the UCam and extrusion through the mNCX. The UCam transports Ca^{2+} down-hill, in an electrochemical diffusion with low temperature dependence (Gunter and Sheu 2009). On the other hand, the mNCX is an antiporter dependent on metabolism, and consequently more influenced by temperature than the UCam. Because of this, it is expected that 10 mmol·L^{-1} Pyr would induce higher $[Ca^{2+}]m$ at low temperatures than at 37 °C. Cortassa et al. (2003) proposed and validated a model to under-

Fig. 7. Changes in cellular Ca^{2+} in rat cardiomyocytes as estimated with confocal microscopy. (*a*) Changes in the cytosolic free [Ca^{2+}] estimated from the changes in relative fluorescence ratio ($\Delta F/F_o$) of Fluo-4 compared with the time before and after perfusing CPG (25 mmol·L^{-1} K^+ – 1 mmol·L^{-1} Ca^{2+}) with or without 10 mmol·L^{-1} pyruvate (CPG+Pyr), followed by the return to control media (C) (n = 9). (*b*) Changes in mitochondrial free [Ca^{2+}] estimated by changes in the relative fluorescence ($\Delta F/F_o$) of Rhod-2 compared with the time during the perfusion with CPG with or without 10 mmol·L^{-1} pyruvate (CPG+Pyr), followed by the return to control media (C) (n = 15). (*c*) Changes in the relative Rhod-2 fluorescence compared with time for the addition of 10 mmol·L^{-1} pyruvate to the C perfusion (C+Pyr) (n = 6).



stand multiple regulations of mitochondrial energy metabolism. They concluded that mitochondrial ATPase flux and protonmotive gradients could be either strongly or slightly increased by an increase in cytosolic Ca^{2+}. The magnitude of the effect depends on the balance between stimulatory effect of Ca^{2+} on the TCA cycle and proton pumping by one hand, and the mitochondrial depolarization due to Ca^{2+}-dependent energy dissipation by the other. This type of modulation could explain why 10 mmol·L^{-1} Pyr induced positive inotropism and high PICR at 37 °C (Figs. 2*c* and 5*b*), but negative inotropism and low PICR at 30 °C (Figs. 2*a* and 4*b*). At this low temperature the [Ca^{2+}]m could be strongly increased, enough to dissipate the gradient and reduce the phosphorylation potential. On the other hand, at 3 mmol·L^{-1} Pyr, the [Ca^{2+}]m is not increased as much, ~~which is~~ enough to induce the TCA cycle and to explain the positive preischemic inotropism and the improved PICR.

In spite of the differences ~~of the~~ inotropism between 3 and 10 mmol·L^{-1} Pyr at 30 °C, both conditions prolonged the contraction (*t*PP and *t*R; Fig. 2), suggesting that Ca^{2+} was cycling among different compartments. This is in line with the reported lengthening of time-to-peak in the Ca^{2+} transient of rat cardiomyocytes with Pyr, which was independent of the cytosolic acidification (Zima et al. 2003). It was also reported that acidosis prolonged relaxation (Toller et al. 2005) and the decay time of both Ca^{2+} transient and caffeine-induced rapid cooling contractures in guinea-pig cardiomyocytes, which was attributed to an acidosis-dependent reduction of Ca^{2+} efflux through the SL-NCX (Terracciano and MacLeod 1994). Some of the effects of Pyr at 10 mmol·L^{-1} and 30 °C, such as the slow relaxation and negative inotropism, could be due to acidosis with the consequent activation of NHE and reverse mode of the SL-NCX. Nevertheless, in previous experiments (not shown), the addition of 5 μmol·L^{-1} KB-R7943 (KBR) to C+Pyr prevented negative inotropism and lusitropism. Although this drug was initially considered a selective blocker of the reverse mode of SL-NCX (Iwamoto et al. 1996), later it was described as a blocker of the UCam at 5 μmol·L^{-1} (Santo-Domingo et al. 2007) and L-type Ca^{2+} channels (Ouardouz et al. 2005). The prevention of the negative inotropism and lusitropism induced by KBR can be explained by the inhibition of the UCam. It suggests that the negative inotropism of Pyr could be partially due to stimulation of mitochondrial Ca^{2+} uptake, which decreases the Ca^{2+} available for contraction. At 3 mmol·L^{-1} Pyr and 30 °C the relaxation was also prolonged without negative inotropism, while at 37 °C and 10 mmol·L^{-1} Pyr the relaxation was not prolonged, probably due to the temperature-induced stimulation of Ca^{2+} removal, mainly by SERCA. The changes in muscle economy (P/H_t) agree with this interpretation, since at 30 °C 10 mmol·L^{-1} Pyr reduced P/H_t, indicating a more expensive contractile state associated to the hampered relaxation and Ca^{2+} accumulation in mitochondria. Another contribution to this energetic expenditure would be the mitochondrial uncoupling associated to the Pyr influx in cotransport with protons (Halestrap and Price 1999). At 3 mmol·L^{-1}, Pyr did not modify the economy, as expected from a lower mitochondrial metabolism in comparison with 10 mmol·L^{-1} Pyr and 30 °C. Finally, at 37 °C 10 mmol·L^{-1} Pyr induced an increase in muscle economy, which agrees with a more efficient relaxation at higher phosphorylation potential. It results from the predominance of SERCA (2 Ca^{2+} per ATP) over the sarcolemmal removal mechanisms (1 Ca^{2+} per ATP) (Ponce-Hornos 1990).

Effects of Pyr on cardioplegic and post-ischemic ventricles

The effect of Pyr on I/R was first assessed in a model of high-[K^+] cardioplegia (CPG) at a relatively low temperature (30 °C). We have reported that this pretreatment increases the post-ischemic contractile recovery (PICR) and muscle economy, reducing the diastolic contracture (ΔLVEDP) with an increase in mitochondrial Ca^{2+}-uptake (Consolini et al. 2004, 2007; Ragone and Consolini 2009; Consolini et al. 2011). During ischemia, H_t abruptly fell to

about zero owing to the quick metabolic inhibition caused by oxygen deprivation, either in ventricles pretreated with CPG or in the non-pretreated hearts. Only the latter developed diastolic contracture (Fig. 4*a*), which may ~~be~~ have been caused by cytosolic Ca^{2+} accumulation associated with the ischemic reduction in ATP production. Although CPG prevented this dysfunction, 10 mmol·L^{-1} Pyr did not increase the cardioprotection of CPG (Fig. 3*b*). Initially, this could be related to the negative inotropism of Pyr demonstrated before the I/R. However, the perfusion of Pyr only during reperfusion did not change the PICR (Fig. 4*b*), suggesting that the pre-ischemic interaction between CPG and Pyr was the prevailing factor. This reduction in PICR was reversed by the presence of KBR during the pretreatment. Considering that this drug has a long-term binding to the UCam, which may remain during ischemia and at least part of reperfusion, the reduced PICR suggests that Pyr also induced stimulation of mitochondrial Ca^{2+} uptake during reperfusion. Energetically, Pyr increased the resting heat rate (H_r) during the CPG arrest (Fig. 3*c*) without changes in resting pressure. This would indicate that Ca^{2+} had not accumulated in cytosol but in the mitochondria and (or) SR. This last destination may be favored by the presence of the UCam blocker KBR. As previously discussed, over a certain threshold the high [Ca^{2+}]m could cause energy dissipation and low phosphorylation potential, which explains the reduced PICR in ventricles pretreated with CPG+Pyr (10 mmol·L^{-1}). KBR not only reversed the drop in PICR, but also increased the post-ischemic economy (P/H_t in Fig. 3*d*), in agreement with the cardioprotection described for this drug (Santo-Domingo et al. 2007). Results in resting cardiomyocytes confirmed that Pyr increased the free mitochondrial Ca^{2+} content (as Rhod-2 signal), more by itself (Fig. 7*c*) than when it had been previously raised with CPG (Fig. 7*b*). In previous work we showed that CPG increased cytosolic and mitochondrial [Ca^{2+}] in rat and guinea-pig cardiomyocytes, owing to a depolarization-induced Ca^{2+} cycling through Ca^{2+} channels, SR, and mitochondria (Consolini et al. 2011; Ragone et al. 2013). However, Pyr reduced the signal of Fluo-4 (cytosolic free [Ca^{2+}]) in CPG (Fig. 7*a*). The results suggest that Pyr would induce a Ca^{2+} movement from cytosol to the mitochondria. Thus the free [Ca^{2+}]m could reach a maximal in cardioplegic cardiomyocytes, consequently inducing Ca^{2+} binding and mitochondrial dysfunction. This mechanism would explain the reduced PICR in the CPG+Pyr-pretreated ventricles and the reversion by KBR.

After evaluating whether Pyr affects Ca^{2+} levels in the SR, we rejected that proposal, since Pyr added to CPG did not change the contractures induced by reperfusing the ventricles with 10 mmol·L^{-1} caffeine – low [Na^+]o (Fig. 6*a*). This result differs from other reports for rat cardiomyocytes, in which 10 mmol·L^{-1} pyruvate increased the 10 mmol·L^{-1} caffeine-induced contractures (Zima et al. 2003). The difference may be that our rat ventricles were exposed to CPG and ischemia before reperfusing to release Ca^{2+} from the SR. In previous work we showed that both CPG (Consolini et al. 1997) and ischemia (Consolini et al. 2007) reduced the SR Ca^{2+} content. There could be compensatory pre-ischemic effects of Pyr. On the one hand, Pyr induces acidosis with a consequent reduction in myofilament sensitivity. On the other hand, Pyr stimulates the phosphorylation potential, which supports SERCA activity and SR Ca^{2+} load. Moreover, the addition of Pyr avoided the effect of KBR in CPG (Fig. 6*a*), suggesting that Pyr reversed the blockade of UCam uptake. The effect of KBR gives evidence of a mitochondrial uptake of a Ca^{2+} fraction released from the SR during the caffeine–low-Na–reperfusion. The mitochondrial uptake was also reported in nonischemic rabbit cardiomyocytes treated with 10 mmol.L^{-1} caffeine and zero Na^+ (Bassani et al. 1993). The heat released during the caffeine–low-Na^+–reperfusion was mainly associated with Ca^{2+} movement through SERCA, SL-Ca-ATPase, and mitochondria, in addition to myofilament ATPase activity, as was previously discussed (Consolini et al. 2007; Ragone et al. 2013). Although Pyr increased the H_r of CPG before ischemia, H_t was not different among the conditions during the caffeine–low-Na^+–reperfusion contractures (Fig. 6*b*). It is probably because the Pyr-induced stimulation of metabolism is a low energetic fraction by comparison with the high exothermic response associated with the contracture and Ca^{2+} movements during reperfusion. An alternative explanation for the loss of synergism between Pyr and CPG as cardioprotectives may be derived from the increase in Ca^{2+} efflux by the SL-NCX and the NHE owing to the stimulation of Na^+–K^+-ATPase in high [K^+]o (Ponce-Hornos et al. 1992). NHE would increase the H^+ gradient, which supports Pyr accumulation in the cytosol symported with H^+. As a consequence, under CPG, Pyr would reach higher intracellular concentrations than under Krebs-C, enough to inhibit RyR (Zima et al. 2003), thus explaining the negative effect on PICR. In conclusion, our results suggest that Pyr accumulation during the pretreatment with CPG induced: (*i*) mitochondrial Ca^{2+} uptake that indirectly reduced the sarcorreticular Ca^{2+} fraction available for the PICR; (*ii*) a possible inhibition of the RyR; and (*iii*) an increase in [Ca^{2+}]m that overcame the beneficial effect on metabolism. All of these mechanisms could explain the antagonistic effects of Pyr and CPG on cardioprotection.

Effects of Pyr on non-cardioplegic post-ischemic ventricles

In support of the conclusion that reduced PICR was due to an interaction between Pyr and CPG, Fig. 4 shows that pretreatment with only 10 mmol·L^{-1} Pyr before ischemia neither affected the PICR nor the energetic output during reperfusion at 30 °C. We saw the same loss of effect when Pyr was added only during reperfusion. Nevertheless, when the solution with 10 mmol·L^{-1} Pyr was present before ischemia and during the whole reperfusion period, there were positive effects on PICR and muscle economy (P/H_t), showing cardioprotection. Several mechanisms that could contribute to pyruvate cardioprotection were reported: the stimulation of aerobic metabolism acting as a substrate (Monteiro et al. 2003; Mallet et al. 2005), its antioxidant capacity (Crestanello et al. 1998; Mallet and Sun 2003), and a stimulation of the SR store (Chen et al. 1998; Zima et al. 2003). Our results in resting cardiomyocytes suggested that in Krebs-C, Pyr increased the [Ca^{2+}]m (Fig. 7*c*), which may contribute to activating the TCA enzymes. The improvement in energetic reserves also explains the reduction in diastolic contracture (ΔLVEDP) during reperfusion in the presence of Pyr, even at 30 °C, as well as the increase in muscle economy (P/H_t). The cardioprotection of Pyr could be sequentially explained by a higher level of ATP before ischemia, an antioxidant effect before and during ischemia, and a post-ischemic influx of Pyr driven by the H^+ gradient after NHE reactivation. Starting with reperfusion, Pyr could reactivate the aerobic metabolism and the phosphorylation potential in 2 ways, acting directly as a substrate (Mallet et al. 2005) and indirectly by stimulating mitochondrial Ca^{2+}-uptake, improving contractility and muscle economy (Heineman and Balaban 1990). When hearts were only pretreated with 10 mmol·L^{-1} Pyr, the pre-ischemic protection was not enough to counteract the deleterious events, which mostly occurred during reperfusion (Di Lisa and Bernardi 2006). This agrees with previous studies of anesthetized dogs with coronary artery occlusion, in which Pyr induced positive inotropism only while it was infused (Mentzer et al. 1989). However, the cardioprotection appeared when ventricles were pretreated with 3 mmol·L^{-1} Pyr in spite of its absence in reperfusion (Fig. 5*a*). As previously discussed, at low [Pyr], acidosis and RyR inhibition must be lower than the stimulation of metabolism and phosphorylation potential, in such a way that the muscle could withstand the deleterious events of reperfusion. On the other hand, when 10 mmol·L^{-1} Pyr was introduced only during reperfusion, it did not reverse the ischemic effects, such as ATP exhaustion and cytosolic Ca^{2+} accumulation (Fig. 4*a*). The cardioprotective effects were strongly improved when 10 mmol·L^{-1} Pyr was present before, during, and after ischemia in hearts at 37 °C (Fig. 5*b*), in accordance with the temperature-

dependent activation of metabolism, phosphorylation potential, and SR Ca^{2+} loading, as discussed above. This cardioprotection of Pyr (high P/H_t) agrees with other reports done on in-vivo or in-vitro models at 37 °C with biochemical indicators, such as the cytosolic phosphorylation potential in rabbits (Chen et al. 1998), reactive oxygen species in rats (Crestanello et al. 1998), and guinea pigs (Bassenge et al. 2000), and infarct size in pigs (Kristo et al. 2004). Thus, the myothermal method provides another tool for evaluating the cardioprotective effects of Pyr.

In summary, to our knowledge this is the first study that simultaneously evaluates the calorimetrical and mechanical effects of Pyr during I/R. This allowed us to estimate the changes in total muscle economy in perfused ventricles in a rat model of stunning. We present a sensitive method to evaluate small changes in energy in myocardial metabolism and Ca^{2+} homeostasis that could occur when contractility has not yet been affected. We found that the cardioprotective effect of Pyr depends on its concentration, temperature, and presence before ischemia and during reperfusion, owing to several cellular mechanisms. Pyr dampens the cardioprotection of a cold cardioplegic high-K^+–low-Ca^{2+} solution, but by itself it is cardioprotective of the stunned heart. These differences in behavior would be a consequence of a dynamic balance among Pyr mechanisms, mainly involving an increase in the phosphorylation potential, a stimulation of mitochondrial Ca^{2+} uptake, and acidosis. This work provides experimental evidence in favor of using Pyr as a cardioprotective blood infusion to prevent stunning in hearts at physiological temperatures, but discourages the use of Pyr as an additive to a hypothermic high-K^+ crystalloid cardioplegia.

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