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Energetic study of cardioplegic hearts under ischaemia/reperfusion and [Ca^{2+}] changes in cardiomyocytes of guinea-pig: mitochondrial role

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Abstract

Aim: To study the role of mitochondria in the recovery of guinea-pig hearts exposed to high-K^+-cardioplegia (CPG) and ischaemia/reperfusion (I/R)

Methods: We measured contractility and heat release in perfused guinea-pig hearts and cytosolic and mitochondrial Ca^{2+} by epifluorescence and confocal microscopy in isolated cardiomyocytes loaded with Fluo-4 or Rhod-2.

Results: In hearts, CPG increased the postischaemic contractile recovery, and this was potentiated by the mNCX blocker clonazepam and the mKATP opener diazoxide, which also prevented the fall in muscle economy. Moreover, CPG prevented the stunning induced by ouabain, which was reduced by clonazepam. In cardiomyocytes, CPG increased fluorescent signals of cytosolic and mitochondrial Ca^{2+}, while the addition of a mNCX blocker (CGP37157) increased cytosolic but reduced mitochondrial [Ca^{2+}]. Ouabain in CPG increased cytosolic Ca^{2+} and resting heat, but the addition of CGP37157 reduced them, as well as mitochondrial Ca^{2+}.

Conclusions: CPG, diazoxide and clonazepam improve postischaemic recovery, respectively, by increasing the Ca^{2+} cycling and by reducing the mitochondrial Ca^{2+} uptake either by uniporter or by mNCX. The mitochondria compete with the leaky sarcoplasmic reticulum (SR) as sink of Ca^{2+} in guinea-pig hearts, affecting the postischaemic contractility. CPG also prevented the ouabain-induced dysfunction by avoiding the Ca^{2+} overload. Ouabain reduced the synergism between CPG and clonazepam suggesting that [Na^+]i and SR load influence the mNCX role.

Keywords Ca^{2+}, calorimetry, cardioplegia, heart, ischaemia–reperfusion, mitochondria.

It is known that mitochondria have a role in the regulation of aerobic metabolism, especially under oxygen deficiency like hypoxia and ischaemia–reperfusion (I/R) (Di Lisa *et al.* 2007 and Graier *et al.* 2007). Nevertheless, during the last years, it has been growing evidence about the role of mitochondria in regulating Ca^{2+} homoeostasis, because these organelles can accumulate Ca^{2+} during stimulation developing beat-to-beat changes in [Ca^{2+}]m (Leisey *et al.* 1993). The beat-to-beat changes in [Ca^{2+}]m have been more or less defined depending on the species (Griffiths 1999, O'Rourke & Blatter 2009). Guinea-pig cardiomyocytes loaded with Indo-1 and Rhod-2 developed beat-to-beat mitochondrial Ca^{2+} transients that symmetrically responded to changes in cytosolic Ca^{2+} transients and to the inhibition of mitochondrial Ca^{2+}

uptake and release (Maack *et al.* 2006). However, these mitochondrial Ca^{2+} changes were only sometimes detected in rat cardiomyocytes depending on methodologies (Andrienko *et al.* 2009, Griffiths 2009, Chen *et al.* 2011). Thus, there are important species-dependent differences in Ca^{2+} mitochondrial regulation, some of which attributed to the $[Na^+]i$, because it modulates mitochondrial Ca^{2+} extrusion via the mitochondrial Na^+/Ca^{2+} exchanger (mNCX) (Maack *et al.* 2006 and Liu *et al.* 2010).

Recently, we have demonstrated the role of mitochondrial Ca^{2+} in the cardioprotection of a high K^+–low Ca^{2+} cardioplegic solution perfused previous to I/R in rat hearts (Ragone & Consolini 2009). The cardioplegic solutions protect the heart from the ischaemic injury evoked during cardiac surgeries, most of them with a high $[K^+]o$ (Chambers & Fallouh 2010). In rat hearts, interventions that reduce $[Ca^{2+}]m$ or block Ca^{2+} extrusion by the mNCX decreased the postischaemic contractile recovery (PICR) with high energetic cost (reduced economy), suggesting that mitochondria contribute with Ca^{2+} to load the sarcoplasmic reticulum (SR) (Ragone & Consolini 2009, Consolini *et al.* 2011). Also, there are several reports suggesting a local Ca^{2+} transfer from the SR to mitochondria as those in rat cardiac mitochondrial fractions with SR particles (García-Pérez *et al.* 2008) or in rat hearts exposed to I/R *in vivo* (Shintani-Ishida *et al.* 2012). The interaction between SR and mitochondria as source or sink of Ca^{2+} during I/R could depend on species. Thus, the SR Ca^{2+} content is larger in rat than in guinea-pig myocytes (Terracciano & MacLeod 1997) and contributes more to the cytosolic Ca^{2+} transient (Bers 2001). Also, the mitochondrial Ca^{2+} uptake in guinea-pig hearts depends on the rate of SR release during the cytosolic transient (Kohlaas & Maack 2010). It is important to consider that human hearts have a Ca^{2+} homoeostasis closer to guinea-pig than to rat hearts (Guo *et al.* 2011). Then, the aim of this work was to study the role of mitochondria in the contractile recovery and Ca^{2+} homoeostasis in guinea-pig hearts exposed to the cardioplegic solution and I/R. Mechano-calorimetrical methodology allows us to evaluate the consequences of I/R while using pharmacological tools to evidence the role of several transporters in both, whole hearts and cardiomyocytes.

Materials and methods

Isolated perfused hearts and mechanical measurements

Adult guinea-pigs (150–300 g weight) were anaesthetized with 25% urethane via i.p. The heart was quickly excised and retrograde perfused with Krebs solution by Langerdorf technique at constant flow of 7 ml min^{-1} g^{-1} kept by a peristaltic pump (Gilson Minipuls, France). A latex balloon was introduced in the left ventricle and connected by a flexible cannula to a Bentley DEL900 pressure transducer. While continuously perfused, the heart with spontaneous beating rate was introduced into the calorimetrical chamber, which was closed and submerged in a bath kept at controlled temperature of 30 ± 0.01 °C as it was previously described (Consolini *et al.* 2007, Ragone & Consolini 2009). Total heat rate (H_t) and the left intraventricular pressure (LVP) were continuously measured after fixing the optimal diastolic volume to obtain isovolumetric contractions. During the experiment, we calculated the maximal pressure development of contraction (P) and the changes in diastolic pressure over the pre-ischaemic condition (ΔLVEDP) in control Krebs (Krebs-C), both in mmHg. During ischaemia (I) and reperfusion (R), P was also expressed as a percentage of the initial P obtained in Krebs-C after stabilization. We also measured the periods of contraction and relaxation before and during R and the normalized rates of contraction and relaxation ($+P/P$ and $-P/P$), but because these parameters were not modified by any protocol, the values are not showed.

Calorimetrical measurements on hearts

We used a flux calorimeter that was previously described (Ponce-Hornos *et al.* 1995, Ragone & Consolini 2009). The thermosensitive units in the internal chamber are sensitive to changes between the inside (heart) and the external (constant) temperatures. We measured the heat rate (H_t in mW g^{-1} of wet heart weight) of hearts in the presence and in the absence of perfusion. For details of calibration and estimation of base lines, see the previous publication (Ragone & Consolini 2009). The signals of both heat rate and intraventricular pressure, respectively, coming from the calorimeter and the pressure transducer were amplified and recorded by a Beckman R511A polygraph and digitalized through a National Instruments P-516 A/D acquisition system. During CPG arresting, the heat rate corresponds to the resting condition (H_r). During R, the recovery of H_t was expressed as percentage of the initial H_t in Krebs-C.

Confocal microscopy in cardiomyocytes

Isolation of cardiac myocytes. Ventricular myocytes were isolated from adult guinea-pigs (200–300 g weight) as previously described (Bridge *et al.* 1990). Once the animal was anaesthetized, 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. After, the solution was changed to Krebs-24-HEPES with 50 μM $CaCl_2$, 0.1 mg mL^{-1} collagenase *P* (Roche) and 0.02 mg mL^{-1} protease XIV (Sigma, St. Louis, MO, USA). After 12 min, the solution was changed again to an enzyme-free 50 μM Ca^{2+} solution. All perfusion solutions were bubbled with O_2 and maintained at 37 °C. Following this, the ventricles were removed and minced. The pieces were gently shaken in a low-Ca^{2+} saline solution for ten minutes and then strained. The isolated cells were stored for up to 6 h in a 1 mM Ca^{2+} HEPES-buffered saline solution.

Confocal microscopy. Isolated guinea-pig cardiomyocytes were loaded with either 12 μM Fluo-4 AM (Molecular Probes/Invitrogen, Carlsbad, CA, USA) during 15 min at room temperature for measuring intracellular free Ca^{2+} signals or 3 μM Rhod-2 AM (Molecular Probes/Invitrogen) during 1 h at 4 °C followed by washup during at least 1 h at 37 °C to load only the mitochondrial compartment (Trollinger *et al.* 1997). After this, cells were placed in a laminin-precoated perfusion chamber. The cells were superfused with Krebs-24 HEPES solution containing 2 mM Ca^{2+} (C) until stabilization, and then, the signals were recorded during the protocols described below. Most cells did not give contracture during the protocol, the ones that did were not considered for the analysis.

Changes in Fluorescence were recorded using a confocal microscope, LSM5 (Carl Zeiss, Jena, Germany) with a 63× oil immersion lens (Numeric aperture, 1.4) (in CVRTI, Salt Lake City, UT, USA, for the protocols of C/CPG/CGP37157) and the Leyca SP5 (Leica Microsystems, Mannheim, Germany) (in UNLP, Argentina, for protocols of C/CPG/CPG+Ouab/CPG+Ouab +CGP37 157 or CPG+Ouab+clonazepam). We analysed the data using the public domain NIH program ImageJ for the Zeiss or the Leica LAS AF Lite v. 2.2.1 software for the Leica results. Results were expressed as the self-ratio emission fluorescence intensity (F/F_0). The changes in F/F_0 over time were calculated by nonlinear adjusting of baseline using Origin 7.0 (OriginLab Corporation, Northampton, MA, USA).

To evaluate cytosolic free Ca^{2+}, Fluo-4-loaded cells were excited at 488 nm, every 20 s during 15 min. Changes in fluorescence emission at wavelengths higher than 505 nm from each cell were monitored through time. To investigate mitochondrial free Ca^{2+}, Rhod-2-loaded cells were excited at 540 nm, every 20 s during 15–20 min. Changes in fluorescence emission (at wavelengths higher than 560 nm) from previously defined areas (files of packed mitochondria) in each cell were monitored through time.

Epifluorescence of cardiomyocytes. To record, intracellular free Ca^{2+} cells were loaded during 15 min at room temperature with 12.5 μM Fluo-4 AM (Molecular Probes/Invitrogen). We placed the cells in a recording chamber mounted on the stage of an inverted microscope (Nikon Diaphot, Nikon Instruments, Melville, NY, USA) precoated with mouse laminin (BD Biosciences). The bath was continuously superfused with a modified 24-HEPES Krebs solution (C). Individual cells were stimulated at a 0.2 Hz frequency either with a field stimulator or by current steps (Axoclamp-2B in bridge mode, MDS Analytical Technologies, Toronto, Canada) to record the membrane voltage. The fluorescence signal was acquired using a custom-designed epifluorescence system. After stabilization, the external solution was replaced with the CPG solution, with or without CGP37157 added. All the signals were acquired and analysed with Clampfit (MDS Analytical Technologies). Results were expressed as the self-ratio emission fluorescence intensity (F/F_0). The changes in fluorescence signal over time were calculated by nonlinear adjusting using Origin 7.0 (OriginLab Corporation).

Solutions and drugs

The composition of control Krebs (Krebs-C) for whole hearts was as follows (in mM): 1 $MgCl_2$, 125 NaCl, 0.5 NaH_2PO_4, 7 KCl, 25 $NaHCO_3$, 2 $CaCl_2$ and 11 dextrose, bubbled with 95% O_2– 5% CO_2. The cardioplegia (CPG) was obtained by changing the solution as follows: 25 KCl, 100 NaCl and 0.5 $CaCl_2$ (in mM). This cardioplegic solution was similar to that previously studied (Ponce-Hornos *et al.* 1995, Consolini *et al.* 2004, 2007, Ragone & Consolini 2009).

For isolation of cardiomyocytes, the solution composition was (in mM): 126 NaCl, 4.4 KCl, 1.0 NaH_2PO_4, 5 $MgCl_2$, 2 HEPES, 22 dextrose, 20 taurine, five creatine, 0.5 Na-pyruvate, adjusted with NaOH to pH 7.4. During the experiment, cardiomyocytes were superfused in Krebs-24-HEPES (C) solution with a composition (in mM): 127 NaCl, 4.4 KCl, 1 $MgCl_2$, 24 HEPES, 2 $CaCl_2$ and 11 dextrose, adjusted with NaOH to pH 7.4. For Fluo-4 experiments, 0.5 mM probenecid was added. CPG was obtained from the Krebs-24-HEPES solution by changing NaCl to 106 mM and KCl to 25 mM and $CaCl_2$ to either 0.5 (CPG) or 1 mM (CPG-1 mM Ca^{2+}).

Clonazepam (Clzp, Saporiti, Argentina) was prepared as an aqueous solution at 10 mM and diluted to 10 μM in CPG for perfusion. Diazoxide (Dzx, Sigma) was prepared in DMSO at 30 mM and diluted in CPG or C to 30 μM. Ouabain (Ouab, Sigma) was prepared as an aqueous solution at 0.25 mg mL^{-1} and diluted to 0.15 μM in Krebs-C or in CPG. KB-R7943

(Tocris-Cookson, Ellisdille, MO, USA) was prepared in DMSO at 12 mM and diluted at 5 μM in CPG. CGP-37157 (Tocris, USA) was prepared in DMSO at 10 mM and then diluted in CPG or C to 1 μM. Nifedipine (Nif, Sigma) was prepared in DMSO at 1 mM and diluted at 1 μM in CPG.

Rhod-2 AM and Fluo-4 AM (Molecular Probes/Invitrogen) were prepared in DMSO and kept frozen in −70°C until use. The loading concentration was 12.5 μM for Fluo-4 and 4 μM for Rhod-2.

Protocols

Effects of cardioplegia on guinea-pig hearts exposed to ischaemia–reperfusion. After 30 min of stabilization in Krebs-C (C), one of the following protocols was applied before ischaemia (Fig. 1): (a1) perfusion with C (30 min); (a2) perfusion with CPG (30 min); (a3) perfusion with CPG (15 min) followed by CPG+ 10 μM Clzp (to selectively block the mNCX) by 15 min (Cox & Matlib 1993, Griffiths *et al.* 1998); (a4) perfusion with C (15 min) followed by C + 10 μM Clzp by 15 min; (a5) perfusion with CPG (15 min) followed by CPG+ 30 μM Dzx (to selectively open the mK_{ATP} channels) by 15 min (Garlid *et al.* 1997, Iwai *et al.* 2000); (a6) perfusion with C (15 min) followed by C + 30 μM Dzx by 15 min; (a7) perfusion with CPG (15 min) followed by CPG + KB-R7943 5 μM [to block the Ca^{2+}-uniporter and the reverse mode of sarcolemmal NCX ($_{SL}$-NCX)] by 15 min (Iwamoto *et al.* 1996, Santo-Domingo *et al.* 2007); (a8) perfusion with CPG (15 min) followed by CPG +1 μM Nif (to block Ca^{2+} L-channels) by 15 min; (a9) perfusion with C + 0.15 μM Ouab (15 min) followed by CPG +0.15 μM Ouab (30 min); (a10) perfusion with C + 0.15 μM Ouab (15 min) following by CPG +0.15 μM Ouab (15 min) and then by CPG + 0.15 μM Ouab + 10 μM Clzp (15 min); and a11) perfusion with C + 0.15 μM Ouab (45 min). After the pretreatment, all the hearts were exposed to 30 min of no-flow ischaemia (I) and 45 min of reperfusion (R) with Krebs-C.

Effects on sarcorreticular Ca^{2+} store. In other series of experiments, the SR Ca^{2+} store at the end of ischaemia was indirectly estimated by reperfusing the hearts with a Krebs-C solution with 10 mM caffeine, 2 mM Ca^{2+} and 36 mM Na^+ (C–caff–low Na) to release Ca^{2+} from SR and prevent the efflux by the $_{SL}$-NCX (Consolini *et al.* 2007). After a stabilization period of about 30 min with C, the pretreatments were carried out by perfusing one of the following solutions as shown in Fig. 1: (b1) C (30 min); (b2) CPG (30 min); (b3) CPG (15 min) and CPG +10 μM Clzp (15 min).

Effects of cardioplegia on the resting energetic performance of hearts. In other series of experiments, the guinea-pig hearts were stabilized at spontaneous beating rate, and then the cardioplegic solution (CPG, with 0.5 mM Ca^{2+}) was applied during 15 min. After that, perfusion was turned back to control condition with Krebs-C, to recover spontaneous beating. Hearts were again exposed to CPG (0.5 mM Ca^{2+}) by 15 min, followed by CPG +1 μM CGP-37157 during

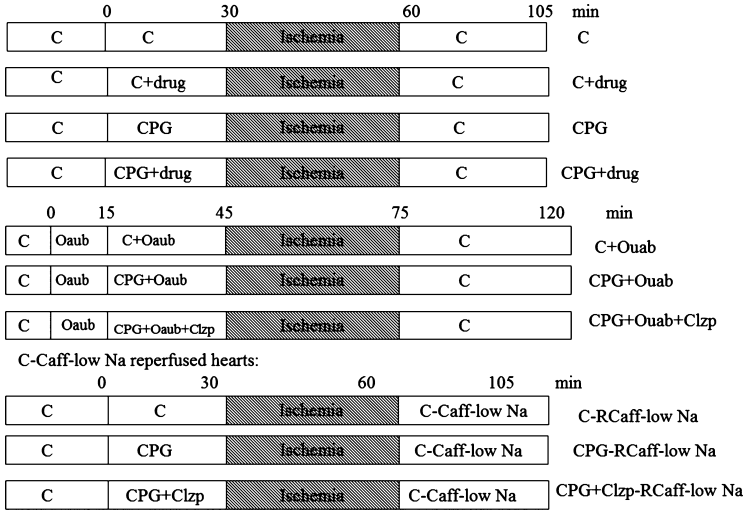

Figure 1 Schematic representation of protocols carried out on the whole hearts of guinea-pigs exposed to no-flow ischaemia (shadow bar) and reperfusion with control Krebs (C, in a and b) or with Krebs–10 mM caffeine–36 mM Na^+ (C–caff–low Na, in Fig. 1c) with several pretreatments: C (control Krebs), CPG (cardioplegia 0.5 mM Ca) and drugs: Clzp (clonazepam), Dzx (diazoxide), Nif (nifedipine), Ouab (ouabain). At above is indicated the time of any perfusion change in minutes. See Materials and Methods for details.

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15 min to inhibit the mNCX (Cox & Matlib 1993) and finally came back to CPG.

Effects of cardioplegia on cytosolic and mitochondrial Ca^{2+} signals of cardiomyocytes. Short protocols (5 min of perfusion in each condition) were carried out with cardiomyocytes in the confocal chamber; we evaluate the changes in Fluo-4 and Rhod-2 fluorescence intensities when perfusing with the following solutions: (d1) Krebs-C, change to CPG (0.5 mM Ca^{2+}), back to Krebs-C; (d2) Krebs-C, change to CPG (0.5 mM Ca^{2+}), then to CPG-1 mM Ca^{2+} and back to Krebs-C; (d3) Krebs-C, change to CPG, followed by CPG +1 μM CGP-37157, back to Krebs-C; (d4) Krebs-C, change to CPG, then to CPG +0.15 μM Ouab, followed by CPG +0.15 μM Ouab +1 μM CGP-37157, back to Krebs-C; (d5) Krebs-C, change to CPG, then to CPG +0.15 μM Ouab, followed by CPG +0.15 μM Ouab +10 μM Clzp, back to Krebs-C.

On the epifluorescence experiments, cardiomyocytes loaded with Fluo-4 were exposed to (e1) Krebs-C, change to CPG, back to Krebs-C; (e2) Krebs-C, change to CPG, followed by CPG +1 μM CGP-37157, back to Krebs-C to evaluate changes in basal free Ca^{2+} level and in the amplitude of the Ca^{2+} transient.

Statistical analysis

Results were expressed in media ± SEM. Multiple comparisons by two-way ANOVA were carried out for P and H_t from the several protocols in whole hearts exposed to I/R, and the results of these ANOVA are showed in Table 1. 'A posteriori' Bonferroni paired tests were carried out among all protocols and are showed in the respective figures when $P < 0.05$. Comparison of more than one group with one variable was carried out by one-way ANOVA and a posteriori Tukey test as shown in Tables 2, 3 and 4. Comparison between two conditions or differences versus zero was carried out with Student's t-test. All the statistical analysis was performed using the GraphPad Prism v.4 software (San Diego, CA, USA), considering significance of $P < 0.05$.

Table 1 Results of two-way ANOVA applied to contractility (%P) and heat release (%H_t) (as% of the initial C value) during the whole protocol of C/treatment/I/R for the following treatment conditions in guinea-pig hearts: C, CPG, CPG + Clzp, C + Clzp, CPG + KB-R7943, CPG + Dzx, C + Dzx, CPG + Nif

Variables of two-way ANOVA	%P	%H_t
By treatment	$F = 127.1$	$F = 17.41$
	DF = 7	DF = 7
	$P < 0.0001$	$P < 0.0001$
By time	$F = 113.7$	$F = 45.33$
	DF = 19	DF = 32
	$P < 0.0001$	$P < 0.0001$
Interaction	$F = 15.64$	$F = 0.9472$
	DF = 133	DF = 224
	$P < 0.0001$	$P = 0.6924$
DF residual	804	1322

(DF: degree of freedom), as described in Figure 1. Results of 'a posteriori' tests are, respectively, shown in Figures 2 and 4.

Animals

The research was carried out with guinea-pigs of both sex and conducted in accordance with the internationally accepted principles for laboratory animal use and care as was established by Good Publishing Practice in Physiology (Persson & Henriksson 2011).

Results

Effects of cardioplegia in guinea-pig hearts exposed to I/R

Mechano-energetic performance during I/R. The guinea-pig ventricles beating with spontaneous heart rate developed a steady maximal intraventricular pressure (P) and total heat release (H_t). In hearts perfused with Krebs-C (C), Ht was decreased from 14.6 ± 1.0 mW g^{-1} to 0.62 ± 0.71 mW g^{-1} ($n = 7$, NS vs. zero) during ischaemia (I). The contractility disappeared in 2–3 min of I with a fall in the diastolic pressure (ΔLVEDP: −10.2 ± 2.2 mmHg), which recovered without contracture during R. Also, both P and Ht recovered during R, respectively, up to 59.1 ± 4.9% of the pre-ischaemic P and 119.6 ± 24.7% of the pre-ischaemic H_t (Fig. 2). The total muscle economy calculated as the P/H_t ratio was reduced during R with respect to the pre-ischaemic (Table 2). The periods of contraction (t_c: 0.19 ± 0.01 s) and relaxation (t_R: 0.33 ± 0.03 s), and the normalized rates of contraction (+P/P: 11.80 ± 0.44 s^{-1}) and relaxation (−P/P: 6.70 ± 0.57 s^{-1}) were not significantly modified by R or by any other protocol (data not shown).

Cardioplegia (Krebs–25 mM K^+–0.5 mM Ca^{2+}) induced the arresting of hearts and the reduction of heat release up to the resting value (H_r) of 5.8 ± 0.5 mW g^{-1}, $n = 9$ (Table 3), which fell to about zero during I. During R, CPG hearts increased the PICR up to 89.0 ± 9.4% of initial P ($P < 0.05$ vs. C hearts, Fig. 2a), while H_t was recovered up to the same degree than C hearts (Fig. 2b). In consequence, in CPG hearts, the total muscle economy (P/H_t) was kept constant between pre-ischaemia and R, preventing the reduction found in C hearts (Table 2).

Table 2 Total muscle economy as P/H_t ratio (in mmHg g mW^{-1}) in the different protocols carried out in guinea-pig hearts during initial *C* condition (and *C*+drug as pretreatment when corresponding) and reperfusion (*R*), both with Krebs-*C*

Condition	*C*/pretreatment	10 min *R*	45 min *R*	ANOVA (*n*)
Control	1.7 ± 0.2	0.6 ± 0.1*	0.8 ± 0.1*	*F*: 14.59 *P* < 0.0002 (7)
CPG	1.1 ± 0.4	1.1 ± 0.3	1.4 ± 0.4	*F*: 0.426 *P* = 0.6579 (9)
CPG +10 μM Clzp	1.9 ± 0.5	2.0 ± 0.4	1.9 ± 0.5	*F*: 0.042 *P* = 0.9589 (8)
C +10 μM Clzp	1.0 ± 0.3/1.0 ± 0.3	0.8 ± 0.1	0.8 ± 0.2	*F*: 0.365 *P* = 0.7795 (4)
CPG +30 μM Dzx	2.2 ± 0.4	2.5 ± 0.7	2.2 ± 0.4	*F*: 0.1043 *P* = 0.9016 (6)
C +30 μM Dzx	0.8 ± 0.1/1.2 ± 0.2	1.2 ± 0.3	1.4 ± 0.4	*F*: 0.517 *P* = 0.6765 (5)
CPG +5 μM KB-R7943	2.5 ± 0.4	2.3 ± 0.6	2.5 ± 0.5	*F*: 0.08862 *P* = 0.9158(5)
CPG +1 μM Nif	0.8 ± 0.4	0.1 ± 0.04	0.1 ± 0.04	*F*: 0.686 *P* = 0.09 (4)
CPG +0.15 μM Ouab	1.6 ± 0.3/3.2 ± 1.1	3.8 ± 1.8	1.8 ± 0.4	*F*: 1.005 *P* = 04113 (6)
CPG +0.15 μM Ouab +10 μM Clzp	3.2 ± 0.6/4.8 ± 1.0	3.7 ± 1.0	3.5 ± 1.0	*F*: 0.5385 *P* = 0.6627 (5)
C +0.15 μM Ouab	2.8 ± 0.7/3.2 ± 1.0	0.25 ± 0.1*	0.9 ± 0.5*	*F*: 4.482 *P* = 0.024 (4)

*ANOVA results and a posteriori tests with $P < 0.01$ vs. initial C condition are shown.

Table 3 Total heat flux during initial beating in Krebs-C (*Ht*) and during the cardioplegic arresting (*Hr*) under the different pretreatments (see Fig. 1) on guinea-pig hearts before ischaemia

Pretreatment	*Ht* ($mW.g^{-1}$) Initial in C	*Hr* ($mW.g^{-1}$) CPG	*Hr* ($mW.g^{-1}$) CPG + drug
CPG	13.8 ± 1.0	5.8 ± 0.5 (*n* = 9)	5.8 ± 0.5 (*n* = 9)
CPG +10 μM Clzp	16.8 ± 2.1	4.6 ± 0.4 (*n* = 7)	5.0 ± 0.6 (*n* = 7)
CPG +30 μM Dzx	10.9 ± 0.9	5.7 ± 0.7 (*n* = 6)	6.1 ± 0.7 (*n* = 6)
CPG +5 μM KB-R7943	14.6 ± 1.6	5.5 ± 0.9 (*n* = 5)	6.8 ± 1.0 (*n* = 5)
CPG +1 μM Nife	12.5 ± 1.7	6.7 ± 0.9 (*n* = 5)	6.1 ± 0.6 (*n* = 5)
CPG +0.15 μM Ouab	16.4 ± 1.8	—	7.3 ± 0.6 (*n* = 6)
CPG +0.15 μM Ouab +10 μM Clzp	16.2 ± 1.7	—	3.5 ± 0.8 (*n* = 5) *
C	14.6 ± 1.0		
C +10 μM Clzp	16.1 ± 1.4		
C +30 μM Dzx	14.7 ± 1,7		
C +0.15 μM Ouab	9.3 ± 1.0		
ANOVA		*P* = 0.306, NS *F* = 1.27 DFn = 4 DF residual: 27	*P* = 0.0158 *F* = 3.067 DFn = 6 DF residual: 36

ANOVA results for each column are shown.
*Bonferroni post-tests: $P < 0.01$ vs. *CPG+Ouab*.

Role of mNCX. To evaluate the role of mNCX, hearts were pretreated with 10 μM Clzp (a selective inhibitor of mNCX) in CPG or *C* before *I/R*. The presence of Clzp did not change H_r during CPG (Table 3) nor altered ΔLVEDP during *I* or *R* regarding those in hearts with C and CPG. Nevertheless, Figure 2a shows that Clzp induced an increase in the PICR in both C hearts (*$P < 0.05$) and CPG hearts

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Table 4 Changes in the resting intraventricular pressure (ΔLVPr) and in heat flux (*ΔHt*) over the end of ischaemia levels obtained during reperfusion with Krebs-C + 10 mM caffeine+36 mM Na^+ (R) of guinea-pig hearts pretreated with C, CPG and CPG+Clzp and turning back to Krebs-C (reversion)

	ΔLVPr (mm Hg)			ΔHt (mW.g^{-1})		
	R-5 min	R-45 min	Reversion	R-5 min	R-45 min	Reversion
C (7)	35.1 ± 8.5	23.2 ± 6.0	22.7 ± 5.4	15.7 ± 2.0	14.2 ± 1.7	14.6 ± 2.4
CPG (6)	46.5 ± 12.5	36.3 ± 8.2	30.7 ± 10.1	11.4 ± 2.5	10.9 ± 2.6	11.2 ± 2.9
CPG–Clzp (6)	24.8 ± 8.0	37.5 ± 8.2	36.6 ± 8.4	7.6 ± 2.0*	6.7 ± 1.7*	6.6 ± 1.7
ANOVA	$F = 1.179$	$F = 1.196$	$F = 0.7922$	$F = 3.539$	$F = 4.07$	$F = 2.587$
	$P = 0.3328$	$P = 0.3280$	$P = 0.4698$	$P = 0.05$	$P = 0.025$	$P = 0.101$

ANOVA results for each column are shown with the tests *a posteriori*.
*$P < 0.05$ vs. *C*.

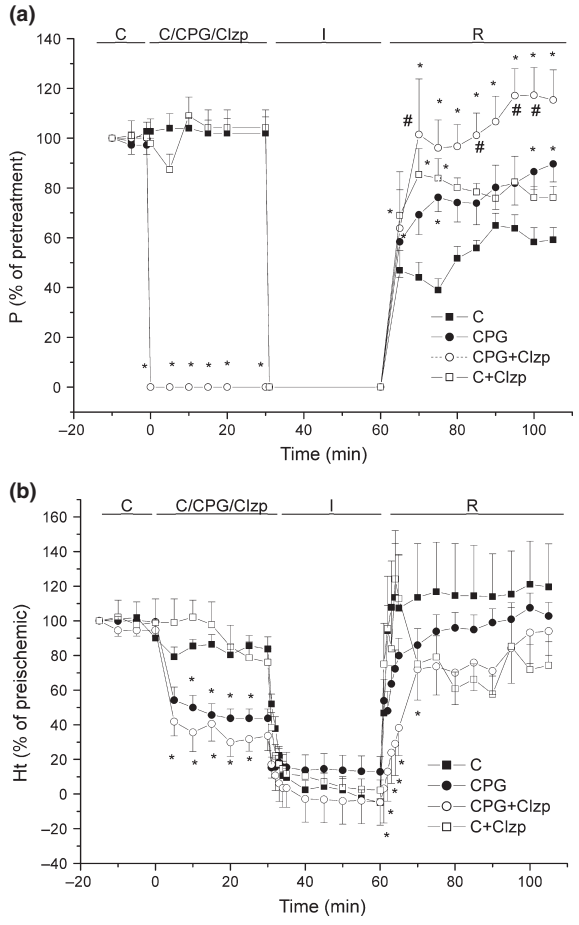

Figure 2 Mechano-energetical performance before, during and after ischaemia in guinea-pig hearts: maximal developed pressure (% of pre-ischaemic *P*) (a) and total heat rate (% of pre-ischaemic H_t) (b) in hearts in normal Krebs (C, $n = 7$) and those pretreated with Krebs–25 mM K^+–0.5 mM Ca^{2+} (CPG, $n = 9$) and/or 10 μM clonazepam (CPG + Clzp, $n = 8$, and C+Clzp, $n = 4$). Results are shown as mean ± SEM, *$P < 0.05$ vs. C and #$P < 0.05$ vs. CPG, by Bonferroni tests *a posteriori* of the two-way ANOVA shown in Table 1.

(#$P < 0.05$), acting synergistically with CPG (CPG + Clzp vs. C + Clzp: $P < 0.05$ between 35 to 45 min *R*). Moreover, Clzp in CPG reduced H_t recovery during the first 5 min of *R*, although later reached the same degree of *C* and CPG hearts (Fig. 2b). The presence of Clzp in CPG prevented the reduction in the economy ratio (P/H_t) during *R*, as well as CPG, avoiding the energetic dysfunction seen in *C* hearts (Table 2).

Effects on the sarcorreticular function of cardioplegic hearts under I/R. To evaluate whether the better PICR of CPG hearts was ought to a higher Ca^{2+} load of SR, hearts pretreated with CPG and CPG + Clzp were exposed to I and then reperfused with Krebs-C with 10 mM caffeine to release Ca^{2+} from SR and a minimal [Na^+] (36 mM) to avoid Ca^{2+} removal via $_{SL}$-NCX (Krebs-C–caff–low Na^+). The R with Krebs-C–caff–low Na^+ induced a diastolic contracture with an increase in H_t (Fig. 3). The contracture induced in hearts pretreated with CPG + Clzp was slower than those in the other protocols. The area under the curve (AUC) of LVPr during R (calculated as changes over I value) was not significantly different among the three conditions (1.029 ± 0.167, 0.933 ± 0.058 and 1.013 ± 0.074 10^5 mmHg min, respectively, for C, CPG and CPG + Clzp pretreatments (ANOVA: $F = 0.213$, $P = 0.8119$). Similarly, the AUC of ΔH_t (over I value) was not significantly different among the three pretreatments [3.71 ± 0.49, 3.10 ± 0.52 and 1.90 ± 0.49 × 10^4 mW g^{-1}.min, respectively, for C, CPG and CPG + Clzp (ANOVA: $F = 3.387$, $P = 0.0593$)], although there was a tendency to the reduction in the last group. In hearts pretreated with C and CPG, the caffeine-induced contracture was slowly reduced during R (Fig. 3a), which was associated with higher heat fluxes than the obtained in hearts pretreated with CPG + Clzp (Fig. 3b, compare the ΔHt in Table 4).

Role of uniporter (UCam) and SL-NCX. To test whether the UCam and the reverse mode of the

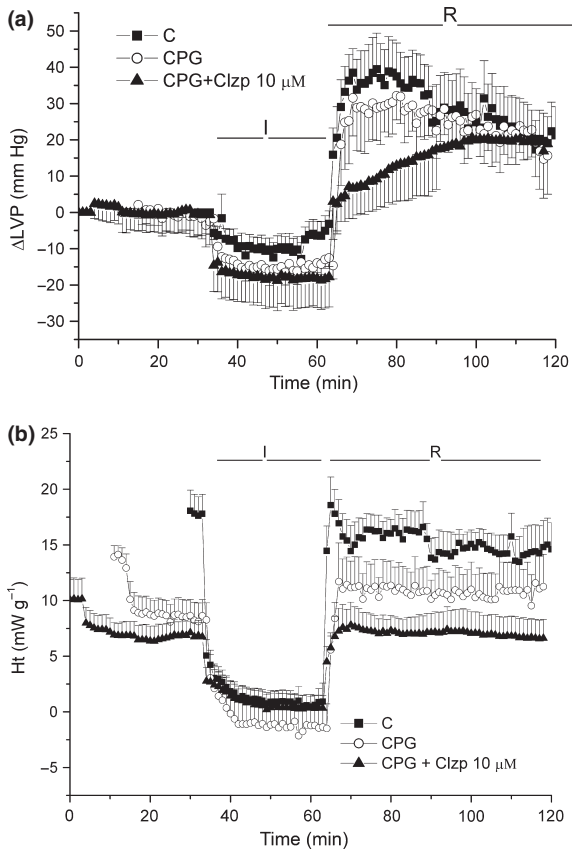

Figure 3 Changes in resting intraventricular pressure (ΔLVP, in a) and absolute total heat rate (H_t, in b) of guinea-pig hearts before and during ischaemia (I) and during the contracture obtained by reperfusion with Krebs–10 mM caffeine–36 mM Na^+ (R) in hearts without pretreatment (C) and pretreated with Krebs–25 mM K^+–0.5 mM Ca^{2+} in the absence (CPG) and in the presence of 10 μM Clzp (CPG–Clzp). Results are shown as mean ± SEM, respectively, of changes in LVP over the pre-ischaemic diastolic pressure in Krebs-C (in mmHg) and absolute H_t (mW g^{-1}).

$_{SL}$-NCX have a role in the cardioprotection of CPG in I/R, 5 μM KB-R7943 was added to the CPG pretreatment. It was demonstrated that this drug blocks both transporters in the same concentration range (Santo-Domingo *et al.* 2007). The presence of KB-R7943 in CPG did not change H_r (Table 3). As well as in C hearts, LVEDP fell during I (in −17.9 ± 8.1 mmHg) and recovered during *R*. Figure 4c,d shows that PICR after CPG + KB-R7943 was similar to that of CPG hearts as well as the recovery of H_t. In consequence, total muscle economy P/H_t was not changed by KB-R7943 (Table 2).

Role of mK_{ATP} channels and L-channels. To evaluate the role of the mK_{ATP} channels opening as a way to reduce the gradient for the UCam, guinea-pig hearts were pretreated with CPG or C + 30 μM Dzx (Garlid *et al.* 1997, Iwai *et al.* 2000). During CPG, Dzx did not affect the H_r (Table 3). During I, Dzx did not alter the fall in LVEDP. During R, Dzx induced an increase in PICR when it was applied in C and potentiated synergistically with CPG (Fig. 4a). Dzx did not change the recovery of H_t with respect to CPG hearts (Fig. 4b) nor changed P/H_t ratio (Table 2).

Because three of the agents (Dzx, KBR7943 and CGP37157) also inhibit L-type Ca^{2+} channels (Ouardouz *et al.* 2005, Thu *et al.* 2006, González *et al.* 2010), we directly assessed this effect on the CPG cardioprotection by adding 1 μM Nif to CPG before I/R. This drug induced a slight but not significant reduction in H_r of CPG (Table 3) without changing LVEDP. But the presence of Nif in CPG strongly reduced the PICR to 16.04 ± 7.98% of the initial *P* but kept a high recovery of H_t as well as in CPG hearts (Fig. 4c,d). Then, Nif showed a tendency to reduce muscle economy estimated as P/H_t (Table 2).

Effects of ouabain during pretreatment with CPG on hearts exposed to I/R. To induce the forward mode of the mNCX, the hearts were pretreated with CPG and 0.15 μM Ouab, a blocker of the Na^+,K^+-ATPase, in the absence and presence of Clzp. Figure 5a shows that in Krebs-C before ischaemia, Ouab induced positive inotropism (up to 157.3 ± 37.3% of the pre-ischaemic *P*, $t = 3.028$, df: 10, $P = 0.0127$), although it did not significantly change the H_t (Fig. 5b). After that, perfusion with CPG+Ouab induced the arresting of hearts, with an increase in H_r regarding hearts with CPG, while the addition of Clzp to CPG + Ouab significantly reduced H_r (Table 3). During I, the presence of Ouab and Clzp did not modify the fall in H_t nor in LVEDP. During R, the presence of CPG prevented the contractile dysfunction induced by Ouab in C, (PICR was increased from 23.4 ± 17.9 to 103.6 ± 20%) without changes in H_t (Fig. 5) by which CPG also prevented the fall in muscle economy (Table 2). The presence of Clzp over CPG+Ouab did not change the contractile nor energetic recoveries (Fig. 5), as neither the P/H_t ratio (Table 3). The three Ouab pretreatments induced diastolic contracture at the start of *R* (ΔLVEDP: +35.9 ± 12.8, +15.0 ± 4.6 and +25.1 ± 9.5 mmHg, respectively, for C + Ouab, CPG + Ouab and CPG + Ouab + Clzp, both $P < 0.05$ vs. zero, ANOVA: $P = 0.27$ among them), which were almost reversed to the end of R in both conditions pretreated with CPG but not in C + Ouab hearts.

Effects of cardioplegia on Ca^{2+} homoeostasis in resting guinea-pig hearts and myocytes

Effects of cardioplegia on the resting energetic of guinea-pig hearts. To evaluate whether the energetic

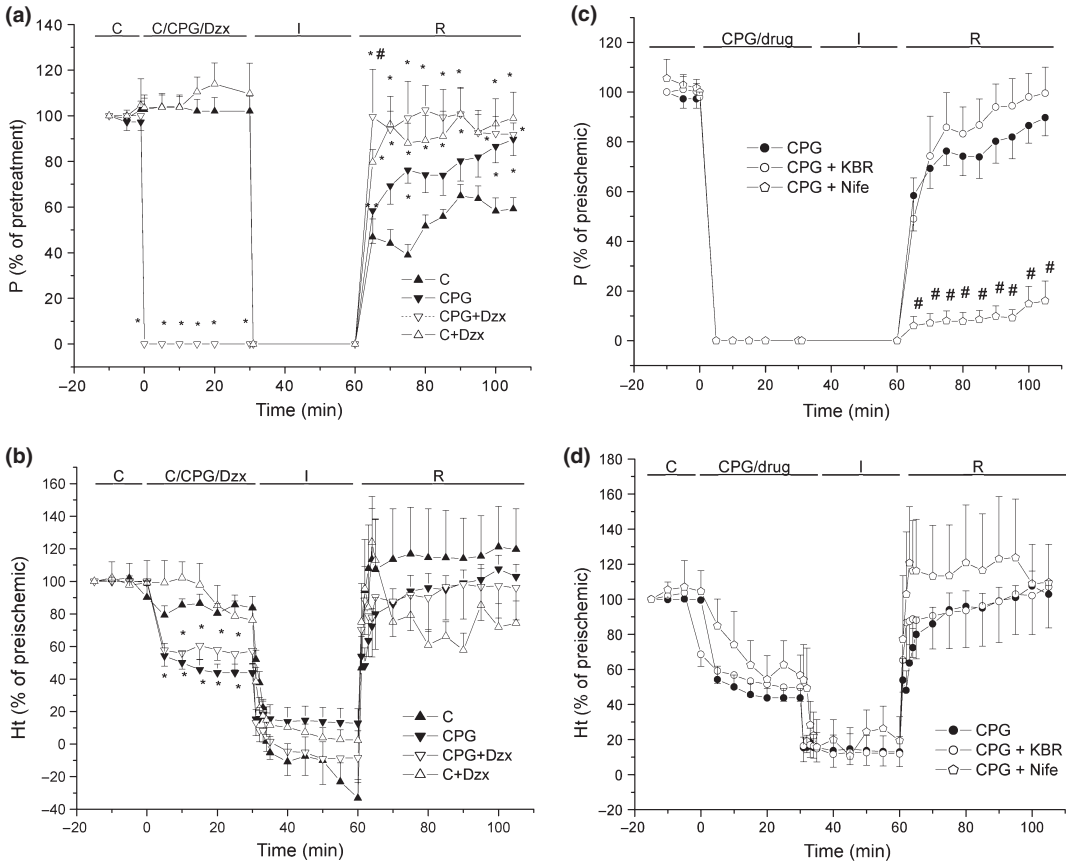

Figure 4 Maximal developed pressure (as% of initial *P*, in a and c) and total heat rate (as% of initial H_t, in b and d) of guinea-pig hearts pretreated with Krebs–25 mM K^+–0.5 mM Ca^{2+} (CPG) or C and the following interventions: 30 μM Dzx (CPG + Dzx, $n = 6$; C + Dzx, $n = 5$), 5 μM KB-R7043 (CPG + KBR, $n = 4$) and 1 μM Nif (CPG+Nif) before, during and after ischaemia. Results are shown as mean ± SEM, *$P < 0.05$ vs. C and #$P < 0.05$ vs. CPG by Bonferroni tests *a posteriori* of the ANOVA shown in Table 1.

effects of CPG were reversible and the consequences of the mNCX blockade during the rest, beating guinea-pig hearts were arrested with CPG (0.5 mM Ca^{2+}), which reduced the H_t (11.30 ± 1.24 mW g^{-1}, $n = 7$) to the resting H_r of 5.93 ± 0.12 mW g^{-1} after 15 minutes and to 6.76 ± 0.98 mW g^{-1} at 1 mM Ca^{2+}, both without changes in LVEDP. Turning back to Krebs-C, hearts began to beat and increased H_t to 8.00 ± 0.08 mW g^{-1} after 15 min. Again, going back to CPG reduced H_r to 5.66 ± 0.71 mW g^{-1} after 15 min, and the subsequent blockade of mNCX with 1 μM CGP37157 in CPG did not significantly change H_r (7.76 ± 1.66 mW g^{-1}) nor LVEDP. Then, H_r remained in 6.48 ± 1.07 mW g^{-1} by turning back to CPG (ANOVA for all H_r in CPG: $P = 0.595$, NS).

Effects of cardioplegia, CGP37157 and ouabain on cytosolic Ca^{2+}. The epifluorescent signals of Fluo-4 showed that CPG (0.5 mM Ca^{2+}) arrested the cardiomyocytes that loose the Ca^{2+}-transients, but it increased the background cytosolic Ca^{2+} (Ca/Cai = 2.1 ± 0.4 times the one in C solution, $n = 12$, Fig. 6a,b). Once back to control Krebs, neither the peak of the Ca^{2+} transients or background Ca^{2+} was completely reversed (Fig. 6b,c). Some cells ($n = 3$) were patched during this protocol to estimate the change in resting membrane potential (E_m) that was reduced from about −75 mV in control C to about −35 to −32 mV in CPG (Krebs–25 mM K^+–0.5 mM Ca^{2+}). The addition of 1 μM CGP37157 (which blocks the mNCX) to CPG further increased background Ca^{2+} (Fig. 6b, $t = 2.495$, $P = 0.0215$, $n = 11$). This effect was also seen when 1 μM CGP37157 was first added to control Krebs (C) and then added to CPG. The final value was not significantly dependent on the order of addition ($t = 1.878$, $P = 0.0719$) (Fig. 6b). Moreover, the presence of CGP37157 in C reduced the Ca^{2+} transient peak to 61.9 ± 8% of the initial value (Fig. 6c), and these ones were partially recovered after CPG and CGP37157 independently of the protocol (Fig. 6c, $t = 0.624$, df: 16, $P = 0.5414$).

In confocal experiments, the Fluo-4 signal was increased by CPG (Ca^{2+} 0.5 mM) and partially

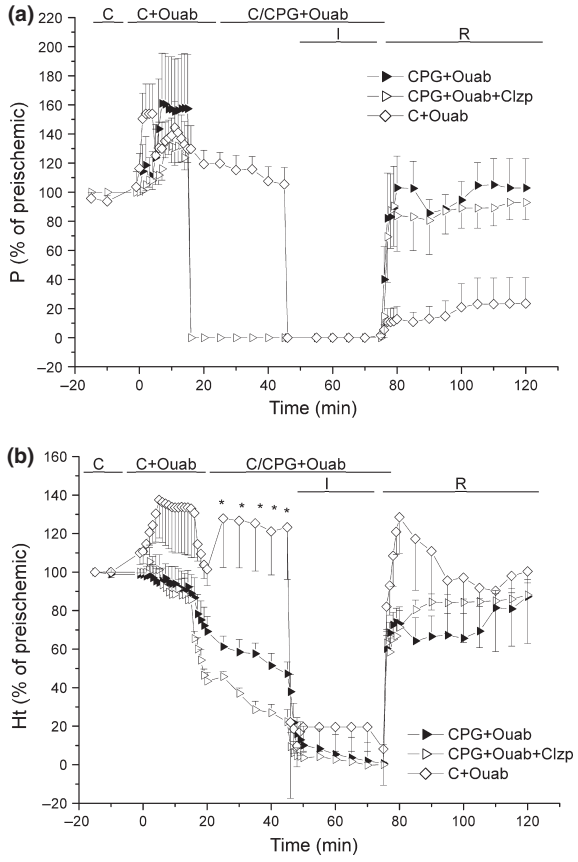

Figure 5 Maximal developed pressure (% of pre-ischaemic *P*) (a) and total heat rate (% of pre-ischaemic H_t) (b) of guinea-pig hearts pretreated with C or with Krebs–25 mM K^+–0.5 mM Ca^{2+} (CPG) in the presence of 0.15 μM de Ouab in the absence (CPG + Ouab, $n = 6$; C + Ouab, $n = 4$) and the presence of Clzp (CPG + Ouab + Clzp, $n = 5$) before, during and after ischaemia. Results are shown as mean ± SEM, two-way ANOVA: *P*%: by treatment: $F = 3.209$, $P = 0.1003$, by time: $F = 30.09$, $P < 0.0001$; for H_t%: by treatment: $F = 121{,}7$, $P < 0.0001$, by time: $F = 26.23$, $P < 0.0001$; and Bonferroni 'a posteriori' tests with $^*P < 0.05$ vs. C

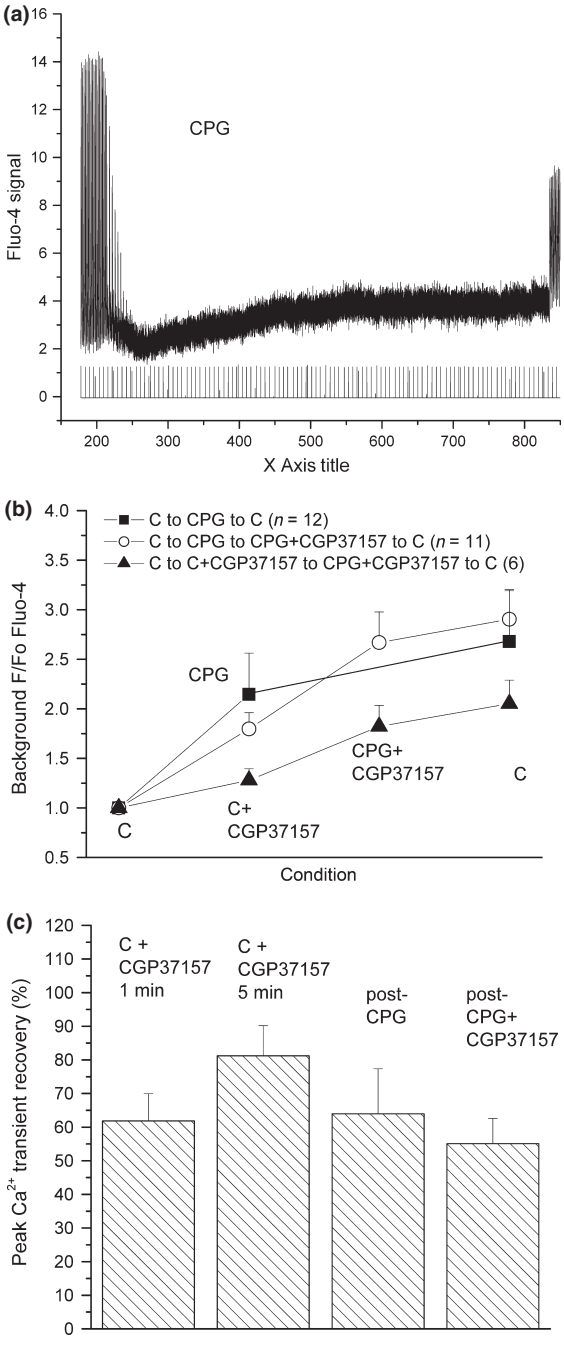

Figure 6 Changes in cytosolic free Ca^{2+} signal estimated by epifluorescence of Fluo-4 in stimulated guinea-pig cardiomyocytes, after the following treatments: Krebs-24-HEPES (C), CPG (Krebs–25 mM K^+–0.5 mM Ca^{2+}), CPG +1 μM CGP-37157 and back to C. In (a) a typical recording, in (b) changes in F/F_0 of background Ca^{2+} signal and in (c) recovery of the peak Ca^{2+} transient (as% of the initial in C). The *x*-axis only shows the sequence of protocols.

reversed back to control medium (C) or further increased in CPG-Ca^{2+} 1 mM (Fig. 7a). On the other side, 1 μM CGP37157 also increased the Fluo-4 signal, either on C or on CPG (Fig. 7b). When Ouab was added to CPG, the Fluo-4 signal was further increased, but the addition of CGP37157 to CPG +Ouab strongly reduced it (Fig. 7c).

Effects of cardioplegia, ouabain and blocking of mNCX on mitochondrial Ca^{2+}. CPG (0.5 mM Ca^{2+}) increased the Rhod-2 signal in cardiomyocytes, and the addition of 1 μM CGP37157 to CPG reduced it (Fig. 8a). The addition of 0.15 μM Ouab to CPG induced a slight increase in Rhod-2 signal, which was also slightly reduced by 1 μM CGP37157 (Fig. 8b). A similar behaviour was obtained when 10 μM Clzp was added to CPG+0.15 μM Ouab (Fig. 8b).

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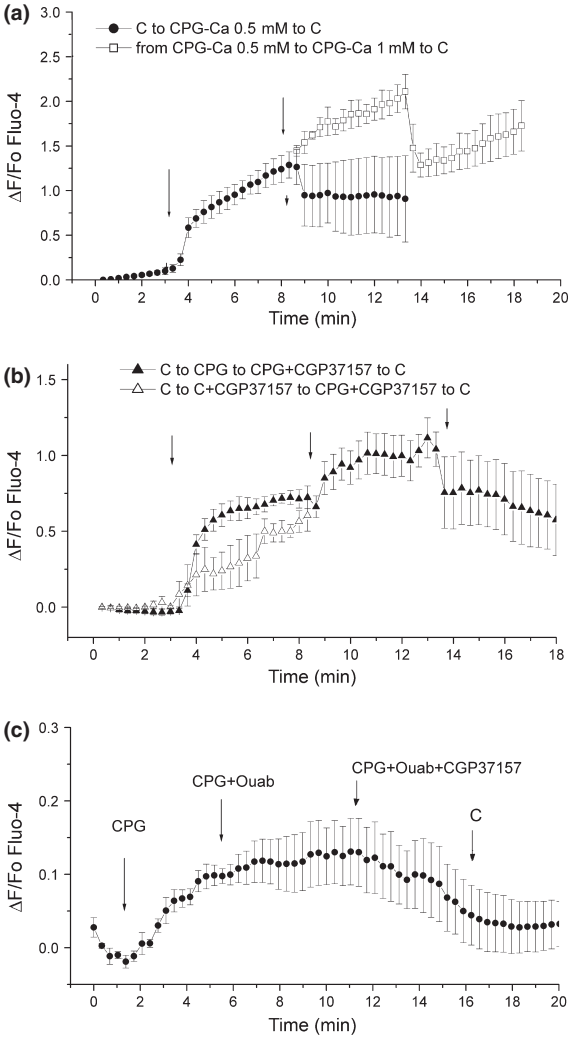

Figure 7 Changes in cytosolic free Ca^{2+} estimated by confocal microscopy of Fluo-4, as change in the fluorescence ratio ($\Delta F/F_0$) in resting guinea-pig cardiomyocytes, after the following treatments: in (a): C was changed to CPG (Ca^{2+} 0.5 mM) (n = 4, filled circle symbols), followed by either CPG-Ca^{2+} 1 mM (white square symbols, n = 2) or back to C (filled circle symbols, n = 2); in (b): C was changed to C +1 μM CGP37157 (n = 2, open triangle symbols) or to CPG (n = 3, filled triangle symbols), both of which were followed by CPG +1 μM CGP37157 (n = 5, filled triangle symbols) and back to C in (c): C was changed to CPG, then to CPG +0.15 μM Ouab and finally to CPG +0.15 μM Ouab +1 μM CGP37157 and back to C (n = 4). The changes of condition occurred at the arrows and were indicated in legends.

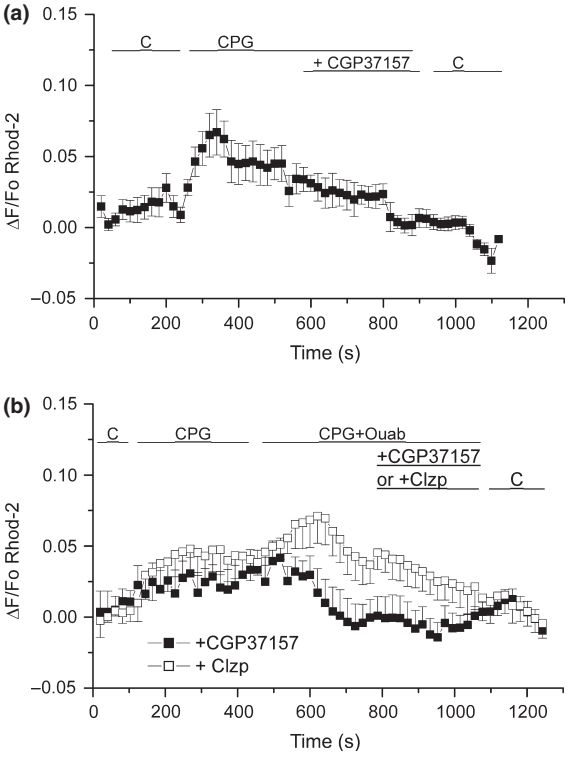

Figure 8 Changes in mitochondrial free Ca^{2+} signal in guinea-pig cardiomyocytes, estimated by confocal microscopy of Rhod-2, as fluorescence ratio ($\Delta F/F_0$) in resting guinea-pig cardiomyocytes, after the following treatments: in (a): C to CPG (Ca^{2+} 0.5 mM), to CPG+1 μM CGP-37157, back to C (n = 6); in (b): two series with C to CPG, to CPG+0.15 μM Ouab, to CPG+0.15 μM Ouab+1 μM CG-37157 (or CPG +0.15 μM Ouab+10 μM Clzp), back to C (n = 9 in the group with CGP37157 in filled square symbols and n = 8 in the group with Clzp in open square symbols). See the interventions at the top of the respective figures.

Discussion

This work shows that in the cardioprotection of a high-K^+ cardioplegia solution (CPG), the role of mitochondrial Ca^{2+} transporters in guinea-pig hearts is different to that of rat hearts, as previously described (Ragone & Consolini 2009). While CPG increased the postischaemic contractile recovery (PICR) in both species, the addition of clonazepam or diazoxide to CPG reduced it in rat hearts but increased it in guinea-pig hearts. Results suggested that in rat hearts, mitochondria contribute with Ca^{2+} to SR load, while in guinea-pig hearts, they mostly contribute to remove cytosolic Ca^{2+}. The behaviour of guinea-pig hearts became in that of rat hearts after adding ouabain to CPG. The mechano-calorimetrical evaluation gave information all over the period of no-flow ischaemia and reperfusion (I/R) showing that CPG prevented the fall in the postischaemic muscle economy in both species.

The whole heart model used in this work was a reversible I/R without infarct but with stunning, as the previously studied in rat hearts (Consolini *et al.* 2007). The H_t during steady-state beating (Table 3) was similar in hearts from both species (Ragone & Consolini 2009). H_t fell during I slower than P,

suggesting that metabolic inhibition was slower than the ischaemic effects of action potential shortening (Cole *et al.* 1991) and inhibition of SR release (Valverde *et al.* 2010). Guinea-pig hearts did not develop diastolic contracture during I/R but fall of LVEDP ought to the loss of perfusion, which might be related to the low SR Ca^{2+} store. Considering the differences between rat and guinea-pig hearts, we aimed to evaluate which was the interaction between SR and mitochondria to regulate Ca^{2+} homoeostasis under CPG and I/R.

Effects of CPG. Perfusion with Krebs–25 mM K^+–0.5 mM Ca^{2+} (CPG) stopped the beating of guinea-pig hearts, with the consequent fall of energetic output from H_t to H_r (about 5.8 mW g^{-1}). In spite of the mechanical resting state, CPG induced an increase in basal $[Ca^{2+}]$i (Fluo-4 signal) ought to the depolarization of cells to −35 mV, in which T- and L-channels are activated (Bers 2001). The basal Fluo-4 signal was further raised by increasing $[Ca^{2+}]$ 0 to 1 mM in CPG (Fig. 7a). Also, *Hr* was slightly increased with 1 mM Ca^{2+} and reduced by the Ca^{2+}-channel blocker nifedipine (Nif) under CPG, suggesting that CPG increased the energetic turnover for Ca^{2+} efflux mechanisms. These Ca^{2+}-dependent energetic changes were lesser in guinea-pig than in rat hearts (Márquez *et al.* 1997, Consolini *et al.* 2011) as it was also showed by Cooper *et al.* (2001) who attributed it to a lower free energy for sarcolemmal NCX in guinea-pig hearts. Also, the mitochondrial Ca^{2+} was raised by CPG because the Rhod-2 signal was slightly increased (Fig. 8) as in rat myocytes (Consolini *et al.* 2011). This agrees with the mitochondrial response to cytosolic Ca^{2+} (Gunter *et al.* 2004). The effects of CPG on background cytosolic Ca^{2+} were not completely reversed neither in stimulated (epifluorescence series) or in resting (confocal series) cardiomyocytes, as neither was the recovery of Ca^{2+} transients and H_t, suggesting that Ca^{2+} homoeostasis and muscle economy were partially affected still in nonischaemic hearts.

Effects of mNCX blockade on $[Ca^{2+}]$i, $[Ca^{2+}]$m and H_r before I/R. As Clzp increased the PICR, we compared the effects of another mNCX blocker CGP37157 under CPG. The epifluorescence and confocal experiments showed that CGP37157 induced an increase in background cytosolic Ca^{2+} during either C or CPG. It is important to consider that CGP37157 is not a selective drug. It increased the RyR activity and reduced the SERCA activity both at EC50 of about 9 μM (Neumann *et al.* 2011), and it blocked Ca^{2+}-channels at IC50 of about 0.27 μM (Thu *et al.* 2006, González *et al.* 2010). The first mechanisms of CGP37157 can explain the increase in background Ca^{2+} (Figs 6b and 7b) and the last the reduction of Ca^{2+} transients (Fig. 6c). Perfusion with CPG +CGP37157 increased the energetic output (ΔH_r: +1.73 mW g^{-1}) of resting hearts without changing LVEDP, suggesting that hearts removed the rise in basal cytosolic Ca^{2+}. Contrarily, the Rhod-2 signal of $[Ca^{2+}]$m was reduced by the addition of CGP37157 to CPG in the confocal experiments (Fig. 8a). Results suggest that mitochondria would uptake Ca^{2+} via mNCX, raising the steady-state of $[Ca^{2+}]$m. Maack *et al.* (2006) also found that CGP37157 reduced the cytosolic Ca^{2+} transients but it increased the mitochondrial ones. The different behaviour of beating myocytes regarding the resting depolarized cells may be related to Ca^{2+} gradients. O'Rourke & Blatter (2009) suggested that cytosolic Ca^{2+} must be raised to about 30 μM in microdomains, in order to be uptaken by the UCam. This level can be reached during the Ca^{2+} transients but not during a resting state even under a depolarizing medium. Nevertheless, high background cytosolic Ca^{2+} could be uptaken by the reverse mode of mNCX ($K_{0.5}$ about 9–10 mM Na^+ and 8–10 nmoles Ca^{2+} mg^{-1} protein or 0.8–1 μmol Ca^{2+} L^{-1}, Gunter *et al.* 2004). This reverse mode was described for other situations as hypoxia (Griffiths *et al.* 1998). Also, the $[Na^+]$i determines the rate of Ca^{2+} extrusion via mNCX in beating cells and explains the species-dependent differences in $[Ca^{2+}]$m transients (Griffiths 1999, O'Rourke & Blatter 2009, Liu *et al.* 2010). When CPG-arrested cardiomyocytes were exposed to ouabain, the effect of CGP-37157 was changed to the reduction of Fluo-4 signal (Fig. 7c). Because ouabain induces an increase in $[Na^+]$i and $[Ca^{2+}]$i, this fall in $[Ca^{2+}]$i could be associated with the CGP-37157 blockade of the few L-channels activated by depolarization or with blockade of the mitochondrial Ca^{2+} extrusion through the mNCX, which was driven by the rise in $[Na^+]$i. The changes in Rhod-2 signal ($[Ca^{2+}]$m) were less defined, but Ouab in CPG developed a slight Ca^{2+} raise followed by a fall, which had a tendency to be further reduced by adding either CGP37157 or Clzp (Fig. 8b). Because Clzp is selective for the mNCX, the results suggest that mNCX would be uptaking Ca^{2+} after 15 min in CPG+Ouab, showing that mNCX can be driven by both the Na^+ and Ca^{2+} gradients.

Cardioprotective mechanisms on I/R

Role of the UCam and L-channels on PICR. KB-R7943 is a blocker of both the UCam (Santo-Domingo *et al.* 2007) and the $_{SL}$-NCX (Iwamoto *et al.* 1996). The addition of KB-R7943 to CPG in whole hearts did not change the pattern of $\%P$,$\%H_t$ nor

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ΔLVEDP during I/R, suggesting that neither the UCam nor the reverse mode of $_{SL}$-NCX influenced the PICR. Nevertheless, an equivalent inhibition on both transporters during CPG and I could be counteracting the effects on cytosolic Ca^{2+} and PICR. Evidences of dual effects of KB-R7943 were found by MacDonald & Howlett (2008). The role of UCam was also evaluated by adding Dzx to CPG, to reduce the ΔΨm by opening mKATP channels with the consequent reduction in the Ca^{2+} uptake driving force. Guinea-pig hearts pretreated with either, CPG + Dzx or C + Dzx improved PICR with respect to *C* hearts preventing the reduction in muscle economy. These results agree with protective effects of Dzx in noncardioplegic hearts from rat (Iwai *et al.* 2000, Murata *et al.* 2001, Ragone & Consolini 2009) and rabbit (Wang *et al.* 2001) and with the synergism between Dzx and high K^+/Mg^{2+} cardioplegia for reducing infarct in rabbit hearts (Toyoda *et al.* 2001). Cardioprotective mechanisms of Dzx on mitochondria have been extensively discussed (Garlid *et al.* 1997, Costa *et al.* 2006, Johansen *et al.* 2011) and include the low mitochondrial Ca^{2+} load by reducing the ΔΨm (Murata *et al.* 2001) and activation of mitochondrial respiration (Riess *et al.* 2008). These energetically opposite effects could explain why H_r was not significantly increased by Dzx over CPG (Fig. 4b).

Because both KB-R7943 and Dzx also inhibit L-type Ca^{2+} channels (Ouardouz *et al.* 2005, González *et al.* 2010) and this could contribute to their effects on PICR, we assessed the effect of the Ca^{2+} channels blocker Nif in CPG before I/R. The strong fall in PICR (Fig. 4c) excludes this mechanism as part of the effects found with KB-R7943 and Dzx. Moreover, results suggest that a reduction in Ca^{2+} influx during CPG would strongly reduce the SR store for PICR.

Role of the mNCX on PICR. The mNCX blocker Clzp increased PICR synergistically with CPG (Fig. 2) and also prevented the fall in muscle economy (P/H_t) in reperfused C hearts (Table 2). Reperfusion with Krebs-C + 10 mM caffeine + 36 mM Na^+ − 2 mM Ca^{2+} showed that Clzp was not increasing the SR load, because the AUC of LVP and H_t did not differ among hearts treated with C, CPG and CPG + Clzp (Fig. 3). Nevertheless, Clzp slowed the caffeine-dependent contracture and avoids the slow relaxation seen in C and CPG hearts, as well as reduced the ΔH_t (see Fig. 3 and Table 4). These results suggest that Clzp may be interfering in the mitochondrial functions as source and sink of Ca^{2+} in a cycling with the SR. The energy associated with the caffeine–low Na contracture (ΔH_t) must include Ca^{2+} removal through transporters except the $_{SL}$-NCX because it was inhibited at 36 mM Na^+. Thus, the *ΔHt* values must contain (i) the futile Ca^{2+} flux through SERCA equivalent to about 1.25 mW g^{-1} (calculated from V_{max} 76 μmol Ca l^{-1} cytosol s^{-1} in guinea-pig hearts or 31 nmol Ca^{2+} g^{-1} s^{-1}, 2 Ca^{2+} by ATP and 80 kJ mol^{-1} ATP, from Bers 2001), (ii) the removal through sarcolemmal Ca-ATPase release 0.2 to 0.3 mW g^{-1} (1 Ca^{2+} by ATP and 2.5 to 3.7 nmol Ca^{2+} g^{-1} s^{-1} from Carafoli 1985, Bers 2001) and (iii) the mitochondrial energy for maintaining Ca^{2+} fluxes, which can be calculated from the reduction induced by blocking the mNCX with Clzp, because *ΔHt* in C and CPG hearts fell to a minimum of 6.7 mW g^{-1} (Table 4). These reductions of about 4.2–9 mW g^{-1} would be associated with the cycling of 52.8–113.2 nmol Ca^{2+} g^{-1} s^{-1} (477 kJ mol^{-1} O_2, 12 H^+: O_2 and 2 H^+: Ca^{2+} from Curtin & Woledge 1978). These Ca^{2+} fluxes are within the range of 32–350 nmol Ca^{2+} g^{-1} s^{-1} estimated from biochemical methods in isolated mitochondria (Carafoli 1985, Arieli *et al.* 2004). These comparisons show that *ΔHt* released during the caffeine–low Na contractures in C and CPG hearts can explain the energy for Ca^{2+} cycling by SERCA, Ca-ATPase and mitochondria, besides that for actomyosin interaction. The increase in PICR induced by Clzp suggests that mitochondria through mNCX and the SR compete as sinks for cytosolic Ca^{2+} during the transients in reperfused guinea-pig hearts.

Effects of ouabain

As in rat hearts the treatment with Clzp in CPG strongly reduced PICR (Consolini *et al.* 2007), we thought that the difference with effects in guinea-pig hearts would respond to the $[Na^+]i$ and SR Ca^{2+} load. When both factors were increased in guinea-pig hearts by inhibiting the Na, K-ATPase with ouabain in CPG, the PICR was improved regarding hearts pretreated only with C+ouabain. Thus, CPG prevented the deleterious effect of Ouab in C hearts exposed to I/R, (Fig. 5). These results suggest that CPG prevents the Ouab-induced mitochondrial and SR Ca^{2+} load by increasing Ca^{2+} removal, as *Hr* of CPG hearts was raised by Ouab (+1.5 mW g^{-1}). This protection is comparable to the prevention of the postischaemic diastolic contracture induced by CPG in rat hearts, which was attributed to Ca^{2+} efflux rise and reduction of SR store (Consolini *et al.* 2004, 2007).

Comparing the results, Ouab reduced the cardioprotection of Clzp in CPG (Figs. 2 and 5). This is suggesting that under the Ouab-induced Ca^{2+} overload, the mNCX plays a lower role in blunting the Ca^{2+} transient and could contribute to extrude Ca^{2+} to cytosol. This is supported by the increase in the Fluo-4 signal ($[Ca^{2+}]i$) evoked in myocytes by Ouab

followed by the reduction induced by CGP37157 (Fig. 7c), which is opposite to the effect in the absence of Ouab (Fig. 7b). On the energetic considerations, Ouab increased resting heat flux (*Hr*) in CPG, while Clzp reduced it, in a similar pattern to that of Fluo-4 and Rhod-2 signals. Depending on whether reverse or forward mode of mNCX predominates, the Clzp-sensitive ΔHr (3.8 mW g^{-1}) may be associated with the Ca^{2+}-dependent mitochondrial metabolism (equivalent to 48.6 nmol Ca^{2+} g^{-1} s^{-1}) or with the removal of cytosolic Ca^{2+} (equivalent to a Ca^{2+} flux between 47.5 nmol Ca^{2+} g^{-1} s^{-1} by sarcolemmal mechanisms and 95 nmol Ca^{2+} g^{-1} s^{-1} if removed by SERCA). These Ca^{2+} fluxes calculated from ΔHr agree to the Ca^{2+} fluxes reported, respectively, for mitochondria, SERCA, $_{SL}$-NCX and Ca-ATPase, as cited before (Carafoli 1985, Bers 2001).

Differences between rat and guinea-pig hearts

Although CPG induced ischaemic cardioprotection in both rat and guinea-pig hearts, the role of mitochondria seems to be different in both species. We previously found that KB-R7943 under CPG reduced the PICR in rat hearts (Consolini *et al.* 2004) but not in guinea-pig hearts. This effect could be due to the KB-R7943 blockade on the RyRs (Barrientos *et al.* 2009) because SR has a higher role in the Ca transient of rat than guinea-pig hearts. On the other hand, although Dzx+C improved PICR in rat as well as in guinea-pig hearts, Dzx + CPG reduced it in rats (Ragone & Consolini 2009) but increased it in guinea-pigs. This is suggesting that Dzx reduces the mitochondrial Ca^{2+} contribution to load the SR store in rat hearts under CPG, while in guinea-pig hearts, Dzx would prevent the mitochondrial Ca^{2+} uptake leaving more Ca^{2+} available for the leaky SR. These differences may be again related to the SR/sarcolemma relative participation in the Ca transient. It was reported that the $_{SL}$-NCX activity is higher in guinea-pig than in rat hearts (Cooper *et al.* 2001) and that SERCA flux in guinea-pig is about one-third of that in rat hearts (Sham *et al.* 1995, Bers 2001). Then, in reperfused guinea-pig hearts, the mitochondria could act as a sink competing with the leaky SR, while in rat hearts, the mitochondria contribute as a source to load the SR. Furthermore, the same pattern of Clzp effect in CPG rat hearts was obtained in guinea-pig hearts exposed to CPG + Ouab, because neither of them improved PICR (Consolini *et al.* 2007). These effects seem related to the conversion of a leaky SR with low $[Na^+]i$ (guinea-pig hearts) in a loaded SR with high $[Na^+]i$ (Ouab-treated guinea-pig hearts as well as rat hearts).

Conclusions

In summary, the mechano-energetic results in whole hearts and the Ca^{2+} signals in cardiomyocytes support the role of mitochondria as sink in guinea-pig hearts exposed to high-K^+ cardioplegia and I/R with stunning. This role was dependent on both $[Na^+]i$ and SR load, which may be driving the mNCX. These factors also explain the differences with the behaviour of rat hearts and ouabain-treated guinea-pig hearts, in which CPG induced the mitochondrial role of source of Ca^{2+}, which affects the contractile postischaemic recovery. CPG reduced the stunning and prevented the diastolic dysfunction, such as that induced by ouabain. Under CPG, the inhibitors of the mNCX Clzp or CGP37157 reduced the mitochondrial Ca^{2+} uptake, leaving more Ca^{2+} available for SR and improving the postischaemic contraction. Also, Dzx improved the postischaemic contractile recovery by reducing the mitochondrial Ca^{2+} uptake by the uniporter. Then, Clzp and Dzx are good cardioprotective agents during exposition to a high K^+ cardioplegia in hearts with a leaky SR as that of guinea-pig. It also offers good perspectives of cardioprotection to other hearts with low SR store as rabbit, dog and human hearts

Conflict of interest

There is no conflict of interest.

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