Mitochondrial role in ischemia–reperfusion of rat hearts exposed to high-K^+ cardioplegia and clonazepam: energetic and contractile consequences

A.E. Consolini, M.I. Ragone, P. Conforti, and M.G. Volonté

Abstract: The role of the mitochondrial Na/Ca-exchanger (mNCX) in hearts exposed to ischemia–reperfusion (I/R) and pretreated with cardioplegia (CPG) was studied from a mechano-calorimetric approach. No-flow ischemia (ISCH) and reperfusion (REP) were developed in isolated rat hearts pretreated with 10 μmol/L clonazepam (CLZP), an inhibitor of the mNCX, and (or) a high K^+ – low Ca^{2+} solution (CPG). Left ventricular end diastolic pressure (LVEDP), pressure development during beats (P), and the steady heat release (H_t) were continuously measured and muscle contents of ATP and PCr were analyzed at the end of REP. During REP, H_t increased more than P, reducing muscle economy (P/H_t) and the ATP content. CPG induced an increase in P recovery during REP (to 90% ± 10% of preISCH) with respect to nonpretreated hearts (control, C, to 64% ± 10%, $p < 0.05$). In contrast, CLZP reduced P recovery of CPG-hearts (50% ± 6.4%, $p < 0.05$) and increased LVEDP in C hearts. To evaluate effects on sarcoplasmic reticulum (SR) function, ischemic hearts were reperfused with 10 mmol/L caffeine –36 mmol/L Na (C – caff – low Na). It increased LVEDP, which afterwards slowly relaxed, whereas H_t increased (by about 6.5 mW/g). CLZP sped up the relaxation with higher ΔH_t, C – caff – low Na produced higher contracture and lower H_t in perfused than in ischemic hearts. Values of ΔH_t were compared with reported fluxes of Ca^{2+}-transporters, suggesting that mitochondria may be in part responsible for the ΔH_t during C – caff – low Na REP. Results suggest that ISCH–REP reduced the SR store for the recovery of contractility, but induced Ca^{2+} movement from the mitochondria to the SR stores. Also, mitochondria and SR are able to remove cytosolic Ca^{2+} during overloads (as under caffeine), through the mNCX and the uniporter. CPG increases Ca^{2+} cycling from mitochondria to the SR, which contributes to the higher recovery of P. In contrast, CLZP produces a deleterious effect on ISCH–REP associated with higher heat release and reduced resynthesis of high energy phosphates, which suggests the induction of mitochondrial Ca cycling and uncoupling.

Key words: calorimetry, ischemia–reperfusion, heart, mitochondria, cardioplegia.

Résumé : On a examiné le rôle de l'échangeur Na/Ca mitochondrial (NCXm) dans des coeurs exposés à une ischémie–reperfusion et prétraités par cardioplégie (CPG), en utilisant une méthode mécano-calorimétrique. On a réalisé une condition d'ischémie (ISCH) et de reperfusion (REP) dans des coeurs isolés de rats prétraités avec 10 μmol/L de clonazépam (CLZP), un inhibiteur de NCXm, et/ou avec une solution riche en K^+ et faible en Ca (CPG). On a enregistré en continu la pression télédiastolique ventriculaire gauche (PTDVG) et le développement de la pression durant les battements (P), ainsi que la libération de chaleur stable (H_t); on a analysé les teneurs musculaires en ATP et en PCr à la fin de la REP. Durant la REP, H_t a augmenté par rapport P, ce qui a réduit l'économie musculaire (P/H_t) et la teneur en ATP. La CPG a augmenté le rétablissement de P durant la REP (à 90 % ± 10 % de la préISCH) comparativement à ce qui a été observé dans les coeurs non prétraités (T, à 64 % ± 10 %, $p < 0{,}05$). à l'opposé, le CLZP a diminué le rétablissement de P dans les coeurs-CPG (50 % ± 6,4 %, $p < 0{,}05$), et augmenté PTDVG dans les coeurs T. Pour évaluer les effets sur la fonction du réticulum sarcoplasmique (RS), on a reperfusé les coeurs ischémiques avec 10 mmol/L de caféine et 36 mmol/L de Na (C – caff – faible Na). La PTDVG a d'abord augmenté puis diminué lentement, alors que H_t a augmenté (d'environ 6,5 mW/g). Le CLZP a accéléré la relaxation et augmenté davantage le ΔH_t que les autres prétraitements. L'association C – caff – faible Na a induit une contraction plus forte et un ΔH_t plus faible dans les coeurs non ischémiques que dans les coeurs ischémiques. On a comparé les valeurs de ΔH_t avec les flux des transporteurs de Ca indiqués dans la littérature; les résultats ont donné à penser que les mitochondries (MIT) pourraient être en partie responsables de la valeur de ΔH_t durant la REP-C – caff – faible Na. Les résultats semblent indiquer que l'ISCH–REP diminue la réserve du RS pour rétablir la contractilité, mais qu'elle induit un déplacement du Ca des MIT vers la réserve du RS. L'ISCH–REP peut aussi éliminer le Ca cytosolique durant les surcharges (ex. caféine) par le biais

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de NCXm et de l'uniport. La CPG augmenterait le cycle du Ca des MIT vers le RS, contribuant ainsi augmenter *P*. À l'opposé, le CLZP produit un effet délétère sur l'ISCH–REP, associé à une plus forte libération de chaleur et à une plus faible resynthèse de phosphates riches en énergie, ce qui suggère l'induction d'un cycle Ca mitochondrial et d'un découplage.

Mots-clés : calorimétrie, ischémie–reperfusion, coeur, mitochondrie, cardioplégie.

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Introduction

Mitochondria play a crucial role in the cardiac dysfunction associated with ischemia–reperfusion (I/R) by affecting the metabolic resynthesis of high-energy phosphates and altering the Ca^{2+}-buffering properties (Gunter et al. 1994; Di Lisa et al. 1998; Takeo and Nasa 1999; Arieli et al. 2004; Di Lisa and Bernardi 2005). The regulation of mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) is done by the interaction of Ca^{2+} influx and Ca^{2+} efflux pathways. Mitochondria can uptake Ca^{2+} through the Ca^{2+} uniporter, which works fast and constantly to sequester Ca^{2+} during physiological conditions. Two mechanisms of Ca^{2+} efflux from mitochondria, the Na/Ca-exchanger (mNCX) and the permeability transition pore (MTP), have been described (Cox and Matlib 1993; Gunter et al. 1994; Arieli et al. 2004; Di Lisa and Bernardi 2005). There are reports showing that a decrease in mNCX activity raises the rate of oxidative phosphorylation (Cox and Matlib 1993). The mNCX regulates the $[Ca^{2+}]_m$, which is adjusted to changes in cytosolic Ca^{2+} pulses (Kiriazis and Gibbs 2000), and indirectly contributes to the metabolic activity associated with cardiac demand (Gunter et al. 1994). In contrast, under Ca^{2+} overload such as under severe hypoxia or ischemia, the mitochondrial Ca^{2+} transporters contribute to cellular injury. Whereas the mNCX seems to function in both directions upon hypoxia–reoxygenation (Griffiths et al. 1998), the MTP was associated with irreversible processes like mitochondrial necrosis and cell apoptosis (Gunter et al. 1994; Di Lisa et al. 1998; Bernardi 1999; Arieli et al. 2004; Di Lisa and Bernardi 2005). Nevertheless, the role of the mNCX on the energetics of a heart under I/R , and thus how Ca^{2+} homeostasis and metabolism affect contractility are still not completely known.

It is well known that cardioplegic solutions are protective against I/R dysfunction, in part owing to induction of cardiac arrest with the consequent low energy consumption (Stowe et al. 2000; Wang et al. 2003). In addition, a high-K^+ solution (cardioplegia, CPG) increases oxygen consumption (Siess 1987) and heat release (Ponce-Hornos et al. 1992; Márquez et al. 1997) during the resting state in well-perfused rat hearts. These effects were related to both an increase in the Na^+, K^+-pump activity (Ponce-Hornos et al. 1992) and a Ca^{2+}- and oxygen-dependent basal heat fraction related to mitochondria (Márquez et al. 1997). Furthermore, the high-K^+ cardioplegia increased another mitochondrial Ca^{2+}- and oxygen-dependent heat fraction released during an isolated contraction (Consolini et al. 1997). In addition, pre-treatment with a high-K^+ medium protected the isolated rat hearts from I/R by avoiding the diastolic contracture and improving the recovery of contractility during reperfusion. In such a response, the sarcoplasmic reticulum (SR) and the sarcolemmal Na/Ca-exchanger (SL-NCX) were involved (Consolini et al. 2004). Therefore, we expected that CPG would increase a Ca^{2+}-dependent mitochondrial activity with the participation of the mNCX, which could contribute to cardioplegic protection. In this work, such a hypothesis was miothermically evaluated.

From studies in isolated mitochondria, it has been proposed that the inhibition of the mNCX by drugs such as verapamil and clonazepam could avoid the mitochondrial Ca^{2+}-release during conditions of Ca^{2+} overload (i.e., hypoxia or ischemia) (Cox and Matlib 1993). This protection was found in isolated cardiomyocytes exposed to hypoxia–reoxygenation, in which clonazepam caused a reversion of the mNCX during hypoxia and was able to decrease the mitochondrial Ca^{2+}efflux during reoxygenation (Griffiths et al. 1998). Nevertheless, neither of those studies evaluated the effect of clonazepam on an intact heart submitted to a real situation of no-flow I/R and its mechano-energetic consequences. Then, the aim of this work was to evaluate from an energetic approach the role of the mNCX on a reversible model of I/R on intact hearts regularly beating or exposed to cardioplegia. The effects of clonazepam were evaluated as a preischemic treatment and during the protection induced by a high-K^+ – low-Ca^{2+} cardioplegia.

The energetics of the heart is highly sensitive to ischemia or hypoxia. Accordingly, online measurements of mechanical and calorimetric performance of a whole isolated beating heart before, during, and after a no-flow ischemia are useful for studying the reversible dysfunction associated with I/R. The calorimetric approach was widely used to estimate changes in exothermic cellular processes during cardiac activity and resting state by the thermopiles method (Loiselle 1987). In particular, online calorimetry for perfused tissues was extensively employed for studying the energetics of Ca^{2+} movement in the steady beating heart (Ponce-Hornos et al. 1982; Ponce-Hornos et al. 1987; Ponce-Hornos et al. 1992), under the transient conditions of a beat (Ponce-Hornos et al. 1995; Consolini et al. 1997; Márquez et al. 1997; Bonazzola et al. 2002), and also during isolated beats under ischemia and hypoperfusion (Consolini et al. 2001). With this methodology, the role of mNCX and the effects of clonazepam on the postischemic recovery of a beating heart exposed or not to a high-K^+ cardioplegia were evaluated in this work. The results indicate that after a period of no-flow ischemia in rat hearts, there is a participation of mitochondria as a donator of Ca^{2+} to the recovery of contractility during reperfusion. Moreover, there is no protective effect of clonazepam upon I/R, neither under pre-treatment with high-K^+ – low-Ca^{2+} cardioplegia nor in its absence.

Methods

Sprague–Dawley rats (fed ad libitum) of both sexes (200–

250 g) were heparinized (2000 U) and anesthetized with a pentobarbital sodium overdose. The beating hearts were rapidly excised and a retrograde perfusion by the Langendorff method was done as previously described (Ponce-Hornos et al. 1995; Consolini et al. 2001; Consolini et al. 2004). Both atria and right papillary muscles were dissected and spontaneous contractions were prevented by a small cut in the septal wall. A latex balloon was placed into the left ventricle and the muscle was mounted in a frame to be placed into the inner chamber of a calorimetric system, analogous to that described elsewhere (Ponce-Hornos et al. 1982; Ponce-Hornos et al. 1995; Consolini et al. 2001). Ventricles were stabilized at 30 °C while stimulated at 1 Hz through 2 flexible electrodes introduced in the chamber. The latex balloon was connected to a Statham Pb 23 Db pressure transducer for measuring pressure developed during isovolumic contractions. At the end of each experiment, hearts were removed from the calorimeter and some of them were immediately frozen in liquid nitrogen to determine high-energy phosphates contents. The other hearts were weighed in a preweighed vial and dried at 110 °C to constant mass so that the water content could be calculated (80.56% ± 0.65%, $n = 25$). Calorimetric results reported in the present work are expressed as mW/g wet mass and high-energy compounds contents were expressed as μmol/g dry mass. All muscles were arterially perfused at a constant rate (6 mL/min) with a control Krebs solution (C) bubbled with 95% O_2: 5% CO_2 to achieve a pH of 7.3–7.4. As in a previous work (Consolini et al. 2004), the conditions of 2 mmol/L Ca^{2+}, 1 Hz of stimulation rate, and 30 °C were chosen to reach a reversible dysfunction with partial recovery of contractility during reperfusion.

Mechanical and heat measurements

The technique for online measurement of heat production and mechanical activity of isolated heart muscles has been described previously in detail (Ponce-Hornos et al. 1982; Ponce-Hornos et al. 1995; Consolini et al. 1997; Consolini et al. 2001). Briefly, the calorimeter was made of a great mass of copper with an internal chamber that contains 2 insulated ceramic thermoelectrics modules (Melchor Thermoelectrics, Trenton, N.J.) with a total of 254 thermosensitive junctions, similar to one previously described (Ponce-Hornos et al. 1995). The minimum output of the thermosensitive units recorded in the present experiments was higher than 10 μV, whereas the electrical noise was about 1 μV at a maximum gain (1 μV/mm). With this method, it was possible to continuously and simultaneously record the signals of left intraventricular pressure and perfusion pressure (calibrated as mmHg) and heat production (H calibrated as mW). The calorimeter was submerged in a bath at 30 °C, and this temperature was controlled with a heating bath (± 0.03 °C) in which the perfusate was also equilibrated. Perfusion baselines were performed before and after the introduction of the muscles to the chamber. Calorimetric calibration was accomplished by passing a known power (1.5 mW) through an electrical resistance kept inside the calorimeter in both conditions, i.e., perfusion of 6 mL/min and no-flow. Both mechanical and heat outputs were recorded on a Beckmann R511A polygraph of 4 channels and converted to analog-to-digital signals by a National Instruments PC-516, which logged the signals into a computer. Mechanical parameters considered for this study were the resting pressure or left ventricular end diastolic pressure (LVEDP) and the maximal intraventricular pressure development during a contraction (P). From P, first-time derivative for obtaining the maximal rates of contraction ($+P$) and relaxation ($-P$) and the pressure-time integral (PtI) were calculated. The whole contraction was divided into 3 periods as follows: t_{PP}, time to peak pressure measured from the start of contraction to time to maximal P, t_{R1} from P to $-P$ time, and t_{R2} from $-P$ time to the end of muscle relaxation.

Once the muscle was placed in the inner chamber of the calorimeter, a 30-min equilibration period with C solution was allowed to elapse before any experimental intervention. The muscle was stimulated by 5 V / 5 ms square pulses from a 611 Phip & Bird (Richmond, Vir.) stimulator. A muscle was accepted for study if, during the equilibration period, a minimum of 50 mmHg of P developed (at 1 Hz). While the muscle was stimulated, resting pressure was gradually increased until P reached a steady maximum value when increasing the volume of the ventricular balloon in steps. Afterwards, the volume was not modified throughout the experiment. Once the steady value of P was achieved during the initial stabilization, the electrical stimulation was stopped, and after 10–15 min, the resting heat rate (H_r) was recorded in those muscles that did not spontaneously beat. During ischemia, pressure development (P) and total heat release (H_t) from contracting hearts were continuously recorded at slow chart and acquisition speeds (1 Hz logging and 0.5 mm/s of chart). Signals coming from the beats were recorded each 5 min before ischemia and during reperfusion at a high rate (50 Hz logging and 25–50 mm/s) of chart speed.

Metabolic measurements

After freezing the muscles at the end of reperfusion (and some during a preischemic condition), they were stored at –80 °C until ATP, PCr, and their metabolites were measured by an HPLC chromatographic method (Volonté et al. 2004). Frozen hearts were homogenized with 0.4 mol/L $HClO_4$ and 2 mol/L KOH and centrifugated at 3000g at 0 °C for 10 min. The previously filtered supernatant was injected into a Konik KNK 500G chromatograph (Barcelona, Spain). An ion-pairing system was used to quantify ATP, ADP, AMP, Cr, CrP, hypoxanthine, and adenosine (Takeo and Nasa 1999; Volonté et al. 2004). Chromatographic conditions were RP-18 reversed-phase column (250 × 4 mm, 5 μm) and a mobile phase consisting of a mixture of 215 mmol/L potassium acid phosphate, 2.3 mmol/L tetrabutylammonium acid sulphate, 4% acetonytrile, and 0.4% KOH 1 mol/L. Flow rate was 1 mL/min; temperature was 25 °C; injection volume was 20 μL. Detection was measured at 220 nm and parameter of integration included peak height and isocratic elution. Tissue contents were expressed as μmol/g dry mass and the energy charge of ATP (E.Ch.) was calculated from the formula $(ATP + 0.5 \times ADP)/(ATP + ADP + AMP)$, where the symbols represent the muscle content of each compound (Iwai et al. 2002).

Solutions and drugs

The control solution (Krebs-C) was prepared as the following (in mmol/L): 1 $MgCl_2$, 125 NaCl, 0.5 NaH_2PO_4,

7 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, and 6 dextrose, bubbled with 95% O_2 : 5% CO_2. With the use of 7 mmol/L K^+, spontaneous contractions were abolished. As in a previous work (Consolini et al. 2004), a high [K^+] – low [Ca^{2+}] solution was used as a model of cardioplegia (CPG) by changing the following saline concentrations from Krebs-C: 25 mmol/L KCl, 100 mmol/L NaCl, and 0.5 mmol/L $CaCl_2$. In some protocols, reperfusion was done with Krebs that contained 10 mmol/L caffeine, 2 mmol/L Ca^{2+}, and only 36 mmol/L Na^+ (C – caff – low Na^+), which was the minimum external Na^+ shown not to damage the viability in an intact heart (Ponce-Hornos et al. 1987). The solution with low Na^+ was prepared by osmotically replacing 114 mmol/L NaCl in Krebs-C by sacarose.

Clonazepam (Saporiti, Argentina) was added at 10 μmol/L either in Krebs-C or CPG from a 10 mmol/L stock solution prepared in dimethylsulfoxide (reaching 0.1% in Krebs, without effect). Caffeine (ICN, USA) was directly dissolved in Krebs.

Protocols

Effects of CPG and (or) clonazepam on energetics of hearts exposed to I/R

Hearts perfused with Krebs-C were equilibrated at 1 Hz stimulation inside the calorimetric chamber until both pressure development and heat release were steady (about 30–40 min). Then the following 4 basic protocols were each performed in 6 hearts (Fig. 1): Krebs-C, followed by 20 min of pre-treatment with Krebs-C, CPG, CPG with 10 μmol/L clonazepam (CPG–CLZP), or Krebs-C with 10 μmol/L clonazepam (C–CLZP) were perfused. Then, a period of 45 min of no-flow ischemia (ISCH) was applied, followed by a period of 45 min of reperfusion with Krebs-C (REP).

Evaluation of the effect on SR function

The SR contribution to contractility was indirectly evaluated at the end of ischemia by reperfusing hearts with a Krebs solution containing 10 mmol/L caffeine, 2 mmol/L Ca^{2+} and 36 mmol/L Na^+ (C – caff – low Na^+). The following protocols were done (n = 6) as follows (Fig. 1).

Krebs-C followed by 20 min of pre-treatment with either Krebs-C, CPG, CPG–CLZP, or C–CLZP were perfused. Then, a period of 45 min of no-flow ischemia, followed by a period of 45 min of reperfusion with C – caff – low Na were applied. Afterwards, the perfusion with Krebs-C was restored (reversion) for 15 min.

In another series of 4 hearts, the SR function of nonischemic hearts was evaluated in comparison with the ischemic ones. Muscles were pretreated with CPG for 15 min, perfused with the same C – caff – low Na^+ solution for 45 min, and afterwards with Krebs-C for 15 min (reversion).

Statistical analysis

Results were expressed as mean ± SE, and paired Student's t test was used to evaluate differences from 0 (p < 0.05). Multiple comparisons were performed using the 1-way analysis of variance (ANOVA) test followed a posteriori by all paired Tukey's tests (GraphPad Prism v. 4).

Animals

The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as was recommended in the Guide for the Care and Use of Laboratory Animals prepared by the Canadian Council on Animal Care (1993) and the Guide for the Care and Use of Laboratory Animals NIH publication N° 85–23, revised in 1985 and 1996.

Results

Prior to any pre-treatment, ventricles stimulated at 1 Hz developed a maximum P= 86.9 ± 10.0 mmHg and H_t of 15.9 ± 1.6 mW/g (n = 24 for the first series of experiments). When a group of 6 nonpretreated hearts (C) was exposed to an ischemic period, the contractility disappeared within 2–3 min, whereas H_t of the muscle continuously fell. Figure 2*a* shows a typical mechano-calorimetric recording during the ischemia and the start of reperfusion in which H_t was increased as well as the resting pressure (LVEDP). Figure 2*b* compares the effects of pre-treatments with CPG, 10 μmol/L CLZP, or both CPG and CLZP on the LVEDP. During ischemia, the LVEDP of nonpretreated hearts (C-hearts) initially decreased owing to the loss of swell, but afterwards it returned to preischemic value with contracture at about 35 min of ischemia. During reperfusion, the LVEDP increased still more until the heart began to beat after about 5 to 10 min; afterwards it decreased to the preischemic value. In contrast, the pre-treatment with CLZP induced contracture during the entire reperfusion period (Fig. 2*b*). On the other hand, pre-treatment with CPG did not increase LVEDP in either the absence or the presence of CLZP (Fig. 2*b*). Figure 3 shows H_t and the maximal P during contractions of the hearts during the whole protocol of I/R. During ischemia in C-hearts, the initial P (112.8 ± 31.0 mmHg) disappeared, whereas the initial H_t (15.2 ± 3.2 mW/g) was reduced to 5.2 ± 2.5 mW/g (n = 6). Neither the pre-treatment with clonazepam nor the other pre-treatments (CPG and CPG–CLZP) significantly modified the effect of ischemia on H_t (1.32 ± 0.53, 2.5 ± 0.7, and 4.2 ± 1.5 mW/g, respecitively, n = 6; p = 0.3046 among the 4 conditions, NS).

During the reperfusion of C hearts, P and H_t were partially recovered until a maximum of 64% ± 10% and 88% ± 17% of the preischemics, respectively (Fig. 3*a*). In C-CLZP hearts (initial P of 75.3 ± 18.6 mmHg and H_t of 15.3 ± 3.5 mW/g) the recovery of P during reperfusion was scarcely but not significantly lower (46% ± 8% of preischemic) than in C hearts, and neither was significantly different the H_t recovery (99% ± 21% of preischemic, Fig. 3*b*). In hearts pretreated with CPG, the recovery of contractility was improved, since the initial P (97.7 ± 12.6 mmHg) was recovered during reperfusion until 90% ± 10% of preischemic (n = 6, p < 0.01 vs. C hearts) with an increase in the total heat release (initial H_t of 11.8 ± 1.9 mW/g) to 146% ± 18% of the preischemic value (p < 0.05 vs. C hearts, Fig. 3*c*). In another group (initial P of 61.7 ± 8.9 mmHg and H_t of 21.5 ± 3.4 mW/g), the addition of 10 μmol/L CLZP during CPG pre-treatment induced a significant reduction in P recovery during the reperfusion (50.0% ± 6.4% of preischemic, n = 6, p < 0.05 vs. CPG hearts), whereas it did not

change the recovery of H_t (until 90% ± 7.8% of preischemic, Fig. 3*d*).

Since it was not possible to estimate the resting heat during the evolution of I/R in the same ventricles while they were stimulated, the economy of contraction (as the ratio between *P* and the energy of a beat) was not calculated. Nevertheless, an index of the total economy of muscle as the P/H_t ratio (where H_t contains the energy rate of basal and contractile activities) was estimated. Table 1 shows that in all groups the economy decreased at the start of reperfusion and in general CPG induced the higher economy of muscles, whereas CLZP in CPG reduced it. Table 2 shows that ischemia and reperfusion only reduced the pressure–time integral (as well as the maximal *P* of contractions, shown in Fig. 3) but not the maximum rates of contraction and relaxation normalized by *P* (+*P*/*P* and –*P*/*P* ratios) nor the times of contraction and relaxation (calculated as t_{PP} and $t_{R1} + t_{R2}$). Similarly, pre-treatment with CPG, CPG-CLZP, or CLZP alone did not modify the times and rates of contraction. This suggests that they only affect the amount of Ca^{2+} released during contractions but not the kinetics of force development and relaxation.

Effects on ATP and PCr contents

As it has been previously shown (Consolini et al. 2004), pre-treatment with CPG significantly increased ATP content at the end of reperfusion with respect to nonpretreated hearts (C) (Table 3). Nevertheless, it did not modify the energy charge of muscles at the end of reperfusion, which remained at a value similar to that previously reported for preischemic hearts (Consolini et al. 2004). Pre-treatment with 10 μmol/L CLZP reduced ATP and PCr contents and the energy charge at the end of reperfusion under both C and CPG pre-treatments. The ATP and the PCr contents were linearly correlated with the pressure development (*P*) considering the preischemic and reperfused muscles of both C and CPG pre-treatments (for ATP vs. *P*: slope 0.05071 ± 0.01587, intercept 2.647 ± 1.471 μmol/g dry mass, $r = 0.5545$, $n = 25$, $p = 0.004$; and for PCr vs. *P*: slope 0.1489 ± 0.043, intercept 8.27 ± 3.89 μmol/g dry mass, $r = 0.5924$, $n = 24$, $p = 0.0023$) (see Fig. 4). Nevertheless, pre-treatment with CLZP strongly reduced the slope of the linear correlation for ATP vs. *P* (slope: 0.02473 ± 0.0069, intercept: –0.034 ± 0.29 μmol/g dry mass, $r = 0.7474$, $n = 12$, $p = 0.0052$) but induced the loss of linear correlation between PCr and *P* (slope: –0.0135 ± 0.0189, intercept: 6.04 ± 0.79 μmol/g dry mass, $r = 0.22059$, $n = 12$, $p = 0.4909$, NS) (see Fig. 4). Both results suggest that CLZP strongly decreased the mitochondrial resynthesis of high energy phosphate compounds.

Effects on sarcorreticular function

To evaluate whether the decrease in the recovery of *P* induced by CLZP during reperfusion was related to a decrease in a Ca^{2+} contribution from mitochondria to the SR, another group of experiments was performed in which hearts were reperfused with Krebs solution containing 10 mmol/L caffeine to release Ca^{2+} from the SR and 36 mmol/L Na^+ to avoid Ca^{2+} efflux by the sarcolemmal NCX. The reperfusion with that medium (C – caff – low Na^+) induced a diastolic contracture with an increase in heat release (Fig. 5). Table 4 compares the magnitude of contracture (as ΔLVEDP) and the increase in heat release (ΔH_t) with respect to the end of ischemia, both at the beginning and after 45 min of reperfusion. It can be seen that at the beginning, the ΔLVEDP was not significantly changed by the type of preischemic treatment. At 45 min of reperfusion, the contracture was slightly decreased in both C and CPG-hearts, suggesting a slow Ca^{2+} removal. In hearts pretreated with CLZP, the ΔLVEDP became not significantly different from 0 at 45 min of the reperfusion with C – caff – low Na^+ (Table 4). Also, the rate of that relaxation was quicker than in hearts not pretreated with CLZP, as was evidenced by the area under the mean curve of ΔLVEDP during the whole reperfusion period (Fig. 5). These mean areas were decreased from 2038 to 1088 mmHg·min in C and C-CLZP, respectively, and from 2440 to 1466 in CPG and CPG-CLZP, respectively.

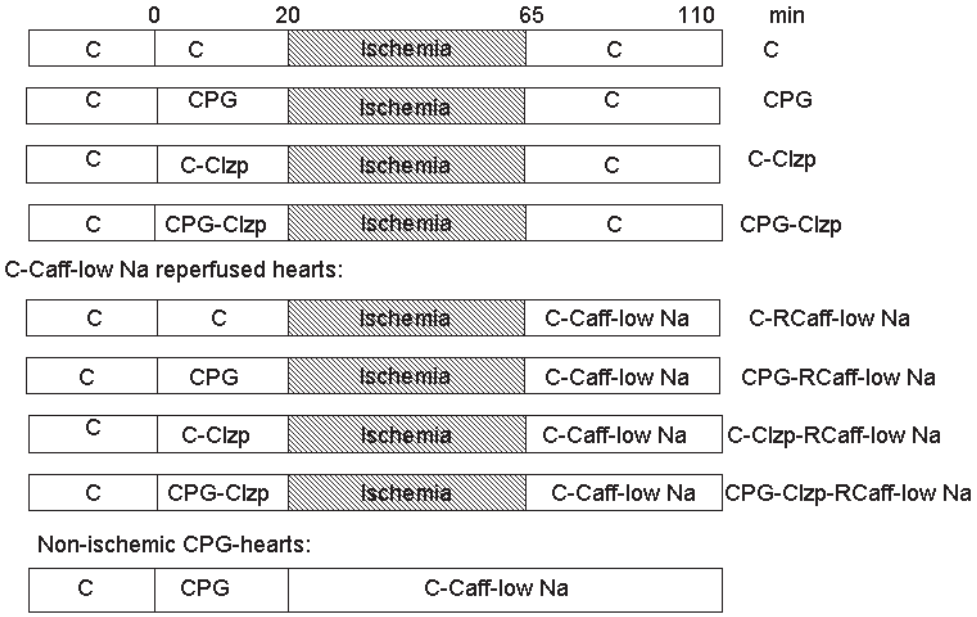
For evaluating whether those changes were due to ischemia or only to C – caff-low Na^+ perfusion, 4 hearts were arrested by CPG and not exposed to ischemia but were perfused with C – caff-low Na^+. Figure 6 shows that the LVEDP increased initially by 42.38 ± 10.51 mmHg, which is lower than the initial ΔLVEDP observed in ischemic hearts. Nevertheless, during the C – caff-low Na^+ perfusion, the ΔLVEDP reached a maximum of +78.36 ± 17.6 mmHg and slowly decreased afterwards until it reached +59.72 ± 20.4 mmHg after 45 min of perfusion. These values of ΔLVEDP and the area under the mean curve of ΔLVEDP (3693 mmHg·min) were higher than those obtained from ischemic hearts, suggesting that ischemia would change the SR function to a quicker release of a lower amount of Ca^{2+}.

The ΔH_t associated with the C – caff – low Na^+ perfusion was significantly different (ANOVA, $p < 0.05$) among the groups of ischemic hearts, in the following order: C+CLZP > C = CPG = CPG+CLZP (see Table 4). The increase in heat release remained high during the entire reperfusion (Fig. 5), suggesting that it was linked to the ATP hydrolysis by myofilaments and by Ca^{2+}-removal mechanisms that support the slow relaxation. On the other hand, although the initial ΔH_t in CPG-nonischemic hearts (9.54 ± 3.04 mW/g, see Fig. 6) was similar to that from the ischemic ones, it was decreased during the whole period of perfusion (until reaching 1.17 ± 4.51 mW/g, NS from 0) despite the stronger contracture. Finally, when the C – caff – low Na^+ perfusion was changed to control-Krebs during 15 min to reverse the effects on SR and NCX, there was a decrease in LVEDP in all muscles associated with an increase in H_t (Fig. 5 and 6). Table 4 shows that neither the ΔLVEDP nor the ΔH_t produced during this reversion were significantly different among the 4 pre-treatments of ischemic hearts. Nevertheless, they were higher in nonischemic hearts (6.16 ± 5.41 mW/g and –34.5 ± 7.9 mmHg), according to the removal of a higher amount of Ca^{2+} from the previous contracture.

Discussion

This work simultaneously analyzed the heat release and contractility of an isolated heart steadily beating and exposed to global I/R for the first time in literature. It also describes the participation of the mitochondrial NCX on contractile dysfunction of I/R and the nonprotective role of clonazepam, a commonly used benzodiazepine that inhibits

Fig. 1. Schema of the different protocols developed. The time scale is indicated at the top (in min) and the type of treatment is at the right of bars (C, Krebs-C; CPG, cardioplegia 25 mmol/L K^+ – 0.5 mmol/L Ca^{2+}; Clzp, clonazepam 10 μmol/L; C – caff – low Na^+, Krebs with 10 mmol/L caffeine, 36 mmol/L Na^+, and 2 mmol/L Ca^{2+}).



this transporter. The calorimetric system is similar to another previously described (Ponce-Hornos et al. 1995) and permitted measures either in the presence or in the absence of perfusion. This point represents an advantage over the thermopiles systems described in the literature (Mulieri and Alpert 1982; Holubarsch et al. 1994; Kiriazis and Gibbs 2000). Although the transient heat release of an isolated contraction during ischemia has been reported previously (Consolini et al. 2001), we have described a more physiological condition because the heart was steadily beating during both ischemia and reperfusion. Figure 2*a* shows that total heat release (H_t) decreased slower than contractility (P) during ischemia. P disappeared after about 3 min of ischemia (or 180 beats), which is different to that previously observed in ischemic hearts only stimulated once every 5 min, which lost their contractility after only 3 beats (in about 15 min) (Consolini et al. 2001). As it was reported, the cessation of contractility is associated to a reduction in Ca^{2+} influx by activation of the K_{ATP} channels during the action potentials upon ischemia (Cole et al. 1991), which supports that the rate of falling in contractility may be related to the heart rate. Comparing with the previous work, it is evident that neither the fall in H_t nor its minimum value during ischemia were affected by differences in heart rate or temperature (2.5 ± 0.75 mW/g in the present results of hearts beating at 1 Hz at 30 °C vs. 1.25 ± 0.03 mW/g previously reported for the hearts beating at 0.0033 Hz at 25 °C) (Consolini et al. 2001). This suggests that the fall in heat release during ischemia must be a manifestation of basal metabolism rather than a consequence of the loss in contractility. In this sense, it has been described that mitochondrial activity depends more on oxygen level than on temperature (Gunter et al. 1994). The fall in H_t during ischemia can be explained by the described deenergization of mitochondria associated with hypoxia as a result of the loss in the respiratory chain function (Di Lisa et al. 1995). From the start of reperfusion, nonpretreated hearts (C) recovered H_t but not P, producing a decrease in the contractile muscle economy (estimated by the P/H_t ratio) during the first 10–15 min. The reduced muscle economy suggests a mitochondrial dysfunction or uncoupling, and agree with our previous results in which the ATP and PCr contents as well as the energy charge were strongly reduced at 5 min reperfusion in CPG- and nonpretreated hearts (Consolini et al. 2004). During the subsequent reperfusion, all the parameters (H_t, ATP, PCr, and E.Ch.) were increased in different degrees. Our results also agree with those of others who have described a mitochondrial reactivation during the start of reperfusion, evidenced by a disproportionately high oxygen consumption with respect to the reduced contractility (Gorge et al. 1991; Neubauer et al. 1988; Di Lisa et al. 1998).

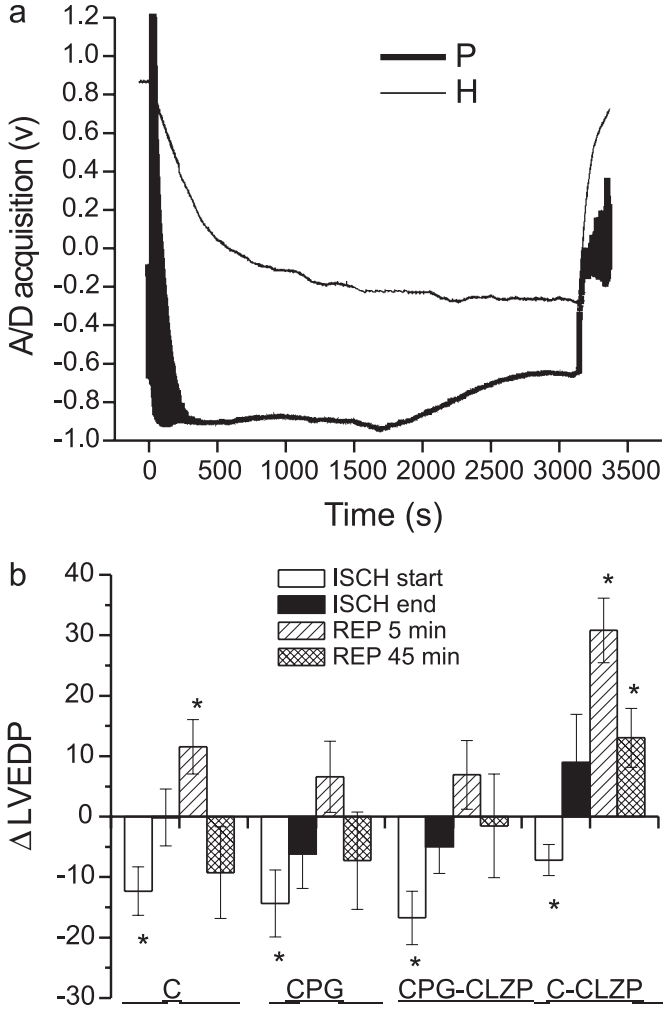
The pre-treatment with CPG increased the muscle economy (P/H_t) and the recovery of P and PtI during reperfusion, with respect to the nonpretreated hearts. Also, CPG induced an increase in ATP content and E.Ch. without changes in the maximum rates of contraction or relaxation ($+P/P$ and $-P/P$). ATP and PCr contents linearly correlated with P in preischemic and reperfused hearts in nonpretreated or those exposed to CPG (Fig. 4), suggesting that the mitochondrial oxidative phosphorylation was adapted to the cardiac demand, estimated by P. Then, the higher recovery in contractility, ATP content and H_t during reperfusion suggests that CPG contributes to increase the Ca^{2+} available in the SR for contraction and stimulates the mitochondrial resynthesis by oxidative phosphorylation.

In previous work done on well-perfused rat hearts, we have described that CPG increases 2 Ca^{2+}- and oxygen-dependent heat fractions sensitive to verapamil, with a different kinetics from that related to Ca^{2+}-channels blockade (Márquez et al. 1997; Consolini et al. 1997). Clonazepam is a selective mNCX inhibitor with a K_i of 7 μmol/L without other effects on the cardiac contractile function and Ca^{2+}

transients (Cox and Matlib 1993; Griffiths et al. 1998). This drug is a benzodiazepine widely used to treat epilepsy, panic attacks, or anxiety, and the knowledge of its effects on a heart suffering a reversible episode of I/R could be of potential clinical importance. Unfortunately, when this drug was added to C or CPG for 20 min before and during ischemia, it induced a decrease in *P*, *P*tI, and H_t recoveries during reperfusion (see Fig. 3). Nevertheless, the drug did not induce changes on the rates of contraction or relaxation (see Table 1) according to the described absence of effects on SL-NCX, SR, or Ca^{2+} channels (Griffiths et al. 1998). Consequently, the effect of clonazepam on contractility suggests a role of mitochondria on the Ca^{2+} homeostasis during I/R. On the energetic aspect, clonazepam induced a decrease in the ATP and PCr contents and in the E.Ch at the end of reperfusion, with a decrease in the slope of the linear correlation between ATP and *P* and a loss in the correlation of PCr vs. *P* (Fig. 4). These results suggest an inhibitory effect of clonazepam on the oxidative phosphorylation, which exceeds the reduction induced in contractility recovery. The observed changes in the dependence of ATP vs. *P* or PCr vs. *P* show that PCr content was more affected than the ATP content, accordingly with a buffer action of the creatin kinase reaction. It has been shown that when the oxygen level of a heart is gradually reduced, the critical pO_2 is higher for PCr than for ATP contents, reducing the PCr/ATP ratio (Zhang et al. 2001). Our results agree with the higher sensitivity of PCr over ATP and suggest that clonazepam inhibits the mitochondrial respiratory chain. Nevertheless, the reduction in the muscle economy estimated by the *P/Ht* ratio of reperfused hearts suggests that clonazepam induces a mitochondrial uncoupling with the consequent increase in heat release, which in turns would drive to a decrease in the oxidative phosphorylation.

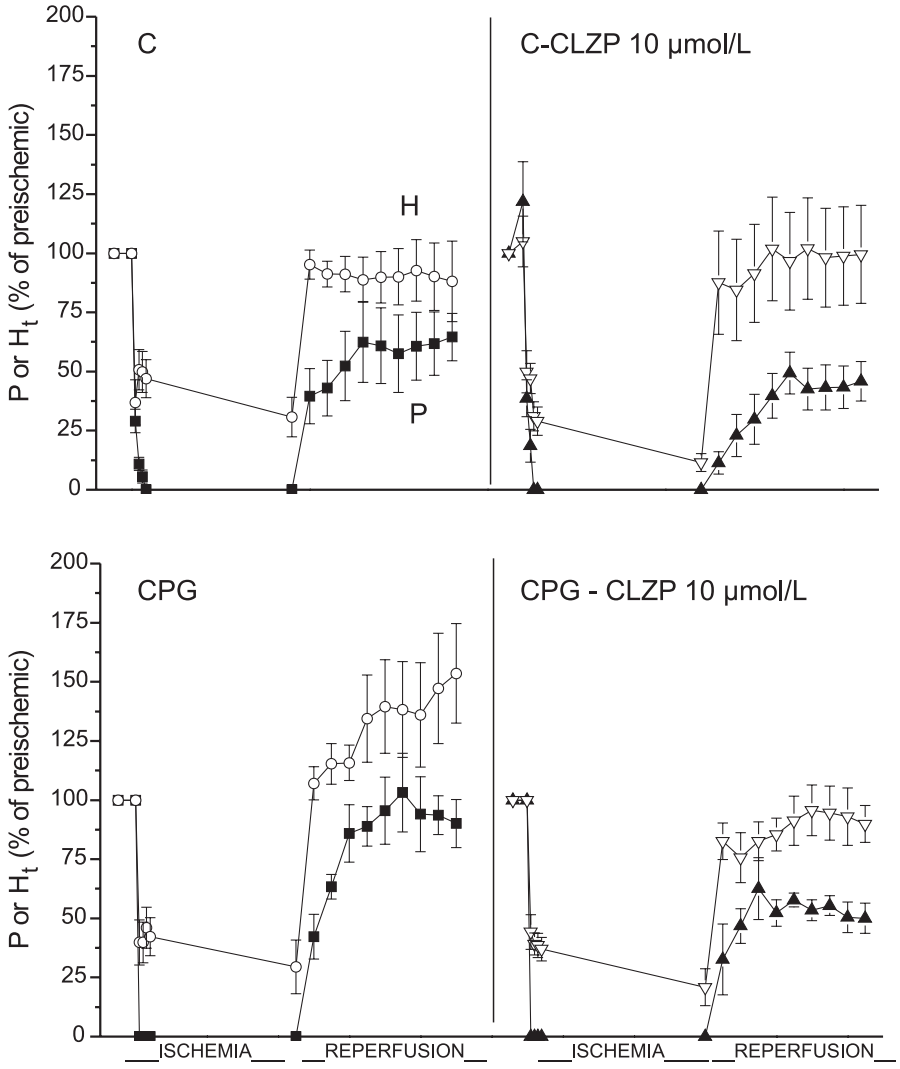
It is known that mitochondrial movements of Ca^{2+} are slow and do not directly contribute to contraction on a beat-to-beat basis (Bernardi 1999; Gunter et al. 1994). Nevertheless, there have been some reports about a slow exchange of Ca^{2+} between mitochondria and SR (Bassani et al. 1993). Also, beat-to-beat transients in mitochondrial Ca^{2+} (Robert et al. 2001) and changes in $[Ca^{2+}]_m$ under hypoxia and reoxygenation in cardiomyocytes (Griffiths et al. 1998) have been measured. When we used 10 mmol/L caffeine as a tool to release Ca^{2+} from SR (Bers 1987) with a low $[Na^+]_o$ of 36 mmol/L to avoid the Ca^{2+} lost via the SL-NCX during reperfusion, a diastolic contracture was obtained, which must be related to the Ca^{2+} content of the SR. As it can be seen in Fig. 5 and Table 4, the contracture (ΔLVEDP) produced by reperfusion with C – caff – low Na^+ was similar for nonpretreated and cardioplegic ischemic hearts, but different from that obtained by perfusing the same solution in nonischemic hearts exposed to CPG (Fig. 6). In nonischemic hearts, the contracture produced by C – caff – low Na^+ was initially lower than in ischemic hearts but slowly increased until reaching a maximum at about 25 min of perfusion. These results suggest that the I/R cycle would change the SR functioning by releasing Ca^{2+} more quickly from the caffeine-sensitive fraction but reducing the whole Ca^{2+} store. This agrees with reports showing that the SR uptake was reduced by ischemia (Abdelmeguid and Feher 1994; Osada et al. 1998) and that ischemia decreased the SERCA gene expression in rabbit hearts (Seehase et al. 2006). A reduction in the SR Ca^{2+} store can also explain the reduced contractility obtained in the first series of hearts pretreated with CPG, which were reperfused with control-Krebs (Fig. 3).

Fig. 2. (*a*) Digital A/D recording of the intraventricular pressure (*P*) and heat release rate (*H*) (both in volts of A/D) of a nonpretreated isolated rat heart exposed to 45 min of ischemia followed by the beginning of the reperfusion period. (*b*) Changes in left ventricular end diastolic pressure (ΔLVEDP, mmHg) of hearts pretreated with control-Krebs (C), cardioplegia (CPG), and the respectives C and CPG in the presence of 10 μmol/L CLZP. The changes were calculated as a difference between the LVEDP at the beginning and at the end of ischemia, or at 5 and at 45 min of reperfusion minus the preischemic LVEDP in C (n = 6, mean ± SE, *p < 0.05 vs. 0).



In both nonischemic and ischemic hearts, the C – caff – low Na^+ perfusion induced an increase in heat release (ΔH_t),which was maintained during the whole process of contracture and slow relaxation. It can be attributed to the active removal of the Ca^{2+} released by caffeine plus the exothermic activity of myofilaments. To evaluate this hypothesis, the ΔH_t measured can be compared with the energy calculated to be used by the different ATP-consuming cellular mechanisms (Ponce-Hornos 1990). Under caffeine treatment, the SR cannot efficiently remove Ca^{2+}, since this

Fig. 3. Percent of maximum pressure development during contractions (P) and total heat release (H_t) obtained during ischemia and reperfusion (% respect to the preischemic value) of hearts pretreated with control-Krebs (C) or cardioplegia (CPG), both in the absence and in the presence of 10 μmol/L CLZP during 20 min before ischemia (n = 6 for each condition, mean ± SE). See the initial absolute values in the text. The $\%P$ values from the reperfusion of CPG–CLZP hearts are significantly lower than those from the CPG-hearts ($p < 0.05$).



drug induces Ca^{2+} release from the SR and prevents the SR Ca^{2+} accumulation (Bers 1987; Rousseau and Meissner 1989). Nevertheless, Ca^{2+} can be cycled through the SR via Ca^{2+}-ATPase with a consequent heat release. Considering the V_{max} of 207 μmol Ca^{2+}·(L cytosol)$^{-1}$·s^{-1} for this pump under caffeine treatment in rat (Bassani et al. 1994), which is equivalent to 85 nmol·g^{-1}·s^{-1} (Bers 2001), with a stoichiometry of 2 Ca^{2+} : 1 ATP, and 80 kJ/mol of resynthesized ATP (Curtin and Woledge 1978), it can be calculated a maximum heat release of 3.4 mW/g. This value is much lower than the initial ΔH_t measured during perfusion with C – caff – low Na^+ in both nonischemic and ischemic hearts (about 9.5 and 8 mW/g, respectively), suggesting that it would be another exothermic mechanism. During the contracture, part of this measured ΔH_t must be associated with the myofilament ATPase activity. With an isometric heat coefficient of 3.6 mN·mm^{-2}·mJ^{-1}·g estimated with the thermopiles method (Mulieri and Alpert 1982), it can be calculated that for maintaining a ΔLVEDP of 70 mmHg (9.3 mN·mm^{-2}) about 0.39 mJ/g (or 0.14 μW/g for the 45 min of perfusion) will be released, which is lower than the measured ΔH_t. Another mechanism that can also contribute to Ca^{2+} removal during the C – caff – low Na^+ contracture is the sarcolemmal Ca^{2+}-ATPase. This pump has a low $K_{0.5}$ and would be functioning near its V_{max} at $[Ca^{2+}]_i$ of about 1.2 μmol/L as reported under 10 mmol/L caffeine (Bers 2001). From its estimated V_{max} of 2.5 to 3.7 nmol Ca^{2+}·g^{-1}·s^{-1} (calculated from Bers 2001 and Carafoli 1985) and the stoichiometry of 1 ATP : 1 Ca^{2+}, it is possible to estimate that the SL Ca^{2+}-pump can release only about 0.3 mW/g. Another important mechanism for removing cytosolic Ca^{2+} is the SL-NCX, but when considering its participation under C – caff – low Na^+ reperfusion, it is expected that it could not efficiently remove Ca^{2+} because of the decreased Na^+ gradient. Consequently, the ΔH_t measured during C – caff – low Na^+ perfusion was higher than the sum of the calculated energetic consumption for the SL and SR Ca^{2+} uptake and myofilaments, suggesting that mitochondria could be contributing to the remaining heat release. Also, the mitochondria are the only available

Table 1. Total economy of muscle (P/H_t) before ischemia (preischemic) and during the reperfusion (Rep) in the 4 pre-treatments of rat hearts studied.

	C	C+CLZP	CPG	CPG+ CLZP
Preischemic	6.81±1.08	6.53±2.12	7.70±0.95	3.58±0.92
Rep 5 min	2.67±0.66	1.68±1.07	3.39±1.21	2.10±0.61
Rep 10 min	2.98±0.68	2.49±1.53	4.28±0.95	2.12±0.61
Rep 15 min	3.60±0.82	2.99±1.75	5.88±1.49	2.64±0.88
Rep 20 min	4.65±1.30	3.65±1.79	5.34±1.22	2.02±0.53
Rep 25 min	4.76±1.48	4.41±1.78	5.66±1.50	2.13±0.50
Rep 30 min	4.77±1.83	3.93±1.87	6.09±1.64	1.96±0.57
Rep 35 min	3.37±0.70	4.14±1.96	5.76±1.56	2.04±0.58
Rep 40 min	3.71±0.82	4.10±1.96	5.75±0.99	1.78±0.47
Rep 45 min	3.98±0.44	4.16±1.76	5.15±1.23	1.83±0.52
Tukey's test vs. C	—	NS	$p < 0.05$	$p < 0.01$
Tukey's test vs. CPG	$p < 0.05$	$p < 0.01$	—	$p < 0.001$
Tukey's test vs. C-CLZP	NS	—	$p < 0.01$	$p < 0.05$

Note: ANOVA: $F = 15.56$, $p < 0.0001$, $r^2 = 0.5646$, $n = 6$ in each group. C, Krebs-control solution; C+CLZP, Krebs-control and clonazepam solution; CPG, high K^+ – low Ca^{2+} solution; CPG+CLZP, high K^+ – low Ca^{2+} and clonazepam solution.

Table 2. Contractile parameters before ischemia (in control Krebs) and after 5, 25, and 45 min of reperfusion (Rep) in the 4 groups of rat hearts studied.

	*P*tI (mmHg·s)	$+P/P$ (s^{-1})	$-P/P$ (s^{-1})	$t_{c1}+ t_{c2}$ (s)	$t_{r1} + t_{r2}$ (s)
Group C					
Preischemic	16.14±6.02	10.75±0.39	7.73±0.56	0.14±0.01	0.35±0.02
Rep 5	7.83±1.96	10.53±0.49	6.81±0.71	0.13±0.002	0.47±0.03
Rep 25	11.33±3.76	10.89±0.84	7.16±1.58	0.13±0.01	0.34±0.04
Rep 45	10.47±2.95	11.25±0.75	8.43±1.42	0.13±0.01	0.31±0.03
Group C+CLZP					
Preischemic	12.79±2.71	10.37±0.69	7.13±0.51	0.15±0.01	0.46±0.07
C+CLZP-preischemic	12.3±2.02	11.55±1.08	7.55±0.69	0.16±0.01	0.48±0.07
Rep 5	3.35±0.4*	11.30±0.57	7.1±0.25	0.14±0.01	0.44±0.06
Rep 25	8.64±4.21	11.81±1.03	8.3±1.19	0.14±0.01	0.41±0.07
Rep 45	5.74±1.88	11.25±1.16	8.17±1.26	0.14±0.01	0.50±0.13
Group CPG					
Preischemic	21.32±3.34	10.7±0.54	6.64±0.3	0.13±0.01	0.34±0.04
Rep 5	9.36±2.62	9.107±1.76	5.7±1.20	0.12±0.01	0.37±0.03
Rep 25	14.64±4.33	9.89±2.08	6.66±1.33	0.13±0.006	0.35±0.05
Rep 45	12.92±3.95	9.73±2.31	6.29±1.47	0.14±0.01	0.31±0.01
Group CPG+CLZP					
Preischemic	11.48±3.95	10.93±0.94	6.46±0.65	0.15±0.03	0.41±0.02
Rep 5	9.83±4.42	9.11±1.76	5.77±1.20	0.14±0.01	0.37±0.05
Rep 25	5.79±1.57	9.89±2.08	6.66±1.33	0.15±0.02	0.39±0.08
Rep 45	4.4±0.88*	9.75±1.06	7.97±0.90	0.13±0.02	0.38±0.07
ANOVA	$F = 1.82$	$F = 0.43$	$F = 0.63$	$F = 0.56$	$F = 0.97$
	$p < 0.043$	NS	NS	NS	NS

Note: C, Krebs-control solution; C+CLZP, Krebs-control and clonazepam solution; CPG, high K^+ – low Ca^{2+} solution; CPG+CLZP, high K^+ – low Ca^{2+} and conazepam solution. *P*tI, pressure-time integral of a contraction; $+P/P$, maximum rate of contraction normalized by P; $-P/P$, maximum rate of relaxation normalized by P; $t_{c1}+ t_{c2}$, time of contraction; $t_{r1} + t_{r2}$ (s), time of relaxation. ANOVA was done among all the conditions for each parameter. *$p <$ 0.05 vs. preischemic condition in each group ($n = 6$) by Tukey's test.

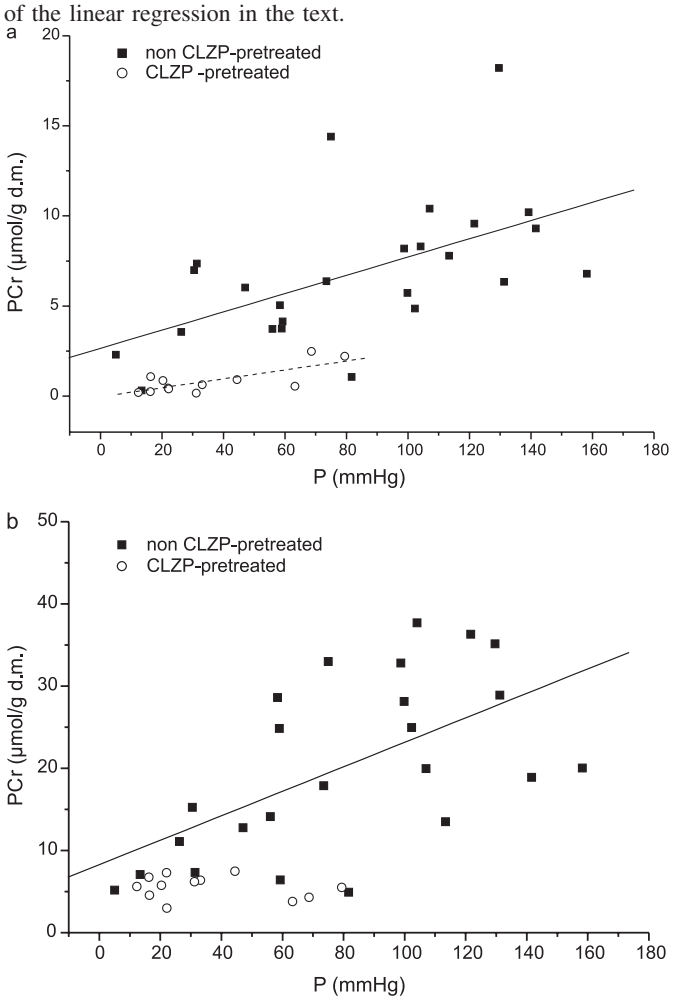
to reduce the contracture under C – caff – low Na^+ perfusion by effectively removing Ca^{2+} from cytosol. Mitochondria can uptake Ca^{2+} by the uniporter with a $K_{0.5}$ of 150–300 nmol/L (Crompton 1990; Bassani et al. 1994) at 1.2 μmol/L Ca^{2+} (under 10 mmol/L caffeine). The reports from the V_{max} of mitochondrial Ca^{2+} uptake are different according to the condition, such as 32 nmol $Ca^{2+}\cdot g^{-1}\cdot s^{-1}$ in physiological Ca^{2+}, 795 nmol $Ca^{2+}\cdot g^{-1}\cdot s^{-1}$ under pathological 10 μmol/L Ca^{2+} (Carafoli 1985), or 350 nmol $Ca^{2+}\cdot g^{-1}\cdot s^{-1}$ for the maximal uniporter flux in rat isolated mitochondria with a blocked MTP (Arieli et al. 2004, by assuming 100 mg protein/g from Carafoli 1985). Assuming the latter V_{max} is nearest to our

Table 3. Tissue contents of high energy compounds and their metabolites (in μmol/g dried mass ± SE) in rat hearts at the end of the reperfusion period, and calculated energy charge after the different preischemic treatments.

Pre-treatment	ATP	ADP	AMP	PCr	Cr	E.Ch.
C (n = 10)	3.73±0.59	2.49±0.41	2.87±0.42	16.57±3.06	22.80±2.32	0.534±0.032
CPG (n = 11)	6.91±0.77*	3.72±0.74	3.88±0.69	18.44±3.88	33.79±5.52	0.608±0.045
CPG+CLZP (n = 5)	0.77±0.095*,†	2.39±0.27	2.86±0.45	6.37±0.48	46.68±11.7*	0.334±0.025*,†
C+CLZP (n = 8)	0.92±0.33*,†	1.81±0.13	1.77±0.12†	4.97±0.45†	24.70±2.89	0.379±0.036*,†
ANOVA	F = 21.21	F = 2.4	F = 2.84	F = 4.73	F = 3.25	F = 9.82
	p < 0.001	NS	p = 0.05	p < 0.01	p < 0.05	p < 0.001

Note: E.Ch: energy charge calculated as (ATP+0.5.ADP)/ (ATP+ADP+AMP); C, Krebs-control solution; C+CLZP, Krebs-control and clonazepam solution; CPG, high K^+ – low Ca^{2+} solution; CPG+CLZP, high K^+ – low Ca^{2+} and clonazepam solution. *p < 0.05 vs. C. †p < 0.05 vs. CPG by Tukey's test.

Fig. 4. Linear correlations obtained between either the (*a*) ATP or the (*b*) PCr muscle contents (in μmol/g dry mass) and the maximal developed pressure (*P,* in mmHg) of muscles not treated with clonazepam (nonCLZP-pretreated muscles) (preischemic untreated muscles and ischemic C-hearts and CPG-hearts reperfused for 45 min) and of hearts pretreated with clonazepam (C–CLZP hearts and CPG–CLZP hearts reperfused for 45 min). See the parameters of the linear regression in the text.



conditions and the energetic equivalent of mitochondria is 12 Ca^{2+}: O_2 and 477 kJ/mol O_2 (Curtin and Woledge 1978; Ponce-Hornos 1990), it is possible to calculate a maximum-associated heat of 13.9 mW/g. This value is higher than the measured ΔH_t under C – caff – low Na^+ perfusion of nonischemic hearts (9.5 mW/g minus about 3.8 mW/g calculated for the other exothermic mechanisms). Then, it is possible that mitochondria contribute to the removal of Ca^{2+} and relax the muscle under C – caff – low Na^+, working at a rate lower than its V_{max}. Our results agree with those that described a mitochondrial Ca^{2+} uptake when the SR was not functional (Bassani et al. 1993).

In ischemic hearts, reperfusion with C – caff – low Na^+ induced a contracture lower and a ΔH_t initially similar but afterwards higher than the respective values measured in nonischemic hearts (see Figs. 5 and 6). Consequently, the I/R cycle may reduce Ca^{2+} release from SR and the higher and maintained ΔH_t may be associated to the mitochondria. In this sense, it is known that the readmission of oxygen by the reperfusion after ischemia increased the mitochondrial respiration and the oxidative phosphorylation (Sako et al. 1988; Di Lisa et al. 1998). Also, the greater rate induced by clonazepam to relax the contracture upon C – caff – low Na^+ when no other mechanism of quick Ca^{2+}-uptake could cause it, suggests that the drug would increase the slow mitochondrial Ca^{2+} removal. Finally, the complete and quick relaxation of muscles was reached when the sarcolemmal Ca^{2+} removal mechanisms were reactivated by returning the perfusion from C – caff – low Na^+ to C levels. Despite myofilaments reducing their energetic consumption, H_t increased again, suggesting that Ca^{2+} was actively removed. Both the SR and SL-NCX can, at that time, contribute to relaxation by effectively removing Ca^{2+}, but the ΔH_t can be mostly attributed to the SL-NCX, because the SR was already consuming energy for the futile Ca^{2+} cycling during the C – caff – low Na^+ perfusion. It can then be estimated that the measured range of 0.8 to 2.4 mW/g for the different conditions would be equivalent to an efflux of 10 to 30 nmol $Ca^{2+}\cdot g^{-1}\cdot s^{-1}$ through the SL-NCX (considering that it is associated to the Na,K-ATPase activity with an stoichiometry of 1 Ca^{2+}: 1 ATP). This calculated value shows a good agreement with the V_{max} of 27 $nmol\cdot g^{-1}\cdot s^{-1}$ reported for the Ca^{2+} fluxes of SL-NCX (Bassani et al. 1994).

The pre-treatment with clonazepam induced a significative decrease in the recovery of contractility during the reperfusion of CPG hearts (Fig. 3) and the area under the ΔLVEDP vs. time curve produced by C – caff – low Na^+ reperfusion both suggest that Ca^{2+} near myofilaments has been reduced. Since this drug selectively inhibits the mNCX (Cox and Matlib 1993; Griffiths et al. 1998) and caffeine releases the SR Ca^{2+} content (Bers 2001), the results strongly suggest that mitochondria can slowly contributes

Fig. 5. Changes in intraventricular pressure (ΔLVEDP, in mmHg, upper) with respect to the preischemic and heat release (H_t, in mW/g, lower) of hearts pretreated with Krebs-control (C), Krebs-control and clonazepam (C–CLZP), high K^+ – low Ca^{2+} (CPG), and high K^+ – low Ca^{2+} and clonazepam (CPG–CLZP) solutions for 20 min before a 45 min period of no-flow ischemia (ischemia) and exposed to reperfusion with Krebs – 10 mmol/L caffeine – 36 mmol/L Na – 2 mmol/L Ca (Rep – C – caff – low Na) for 45 min, followed by changing the perfusion to Krebs-control solution (reversion to C) (n = 6, mean ± SE).

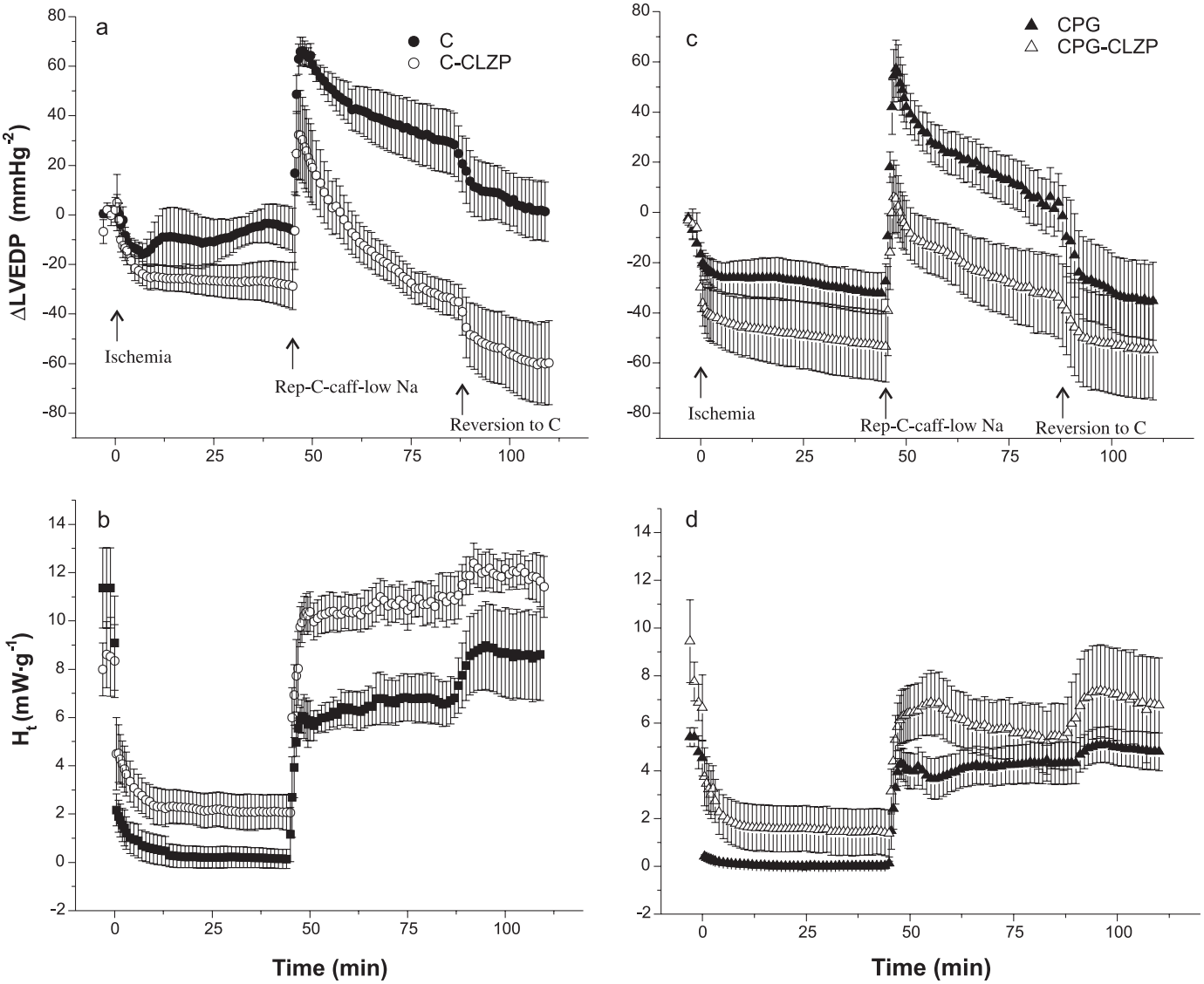
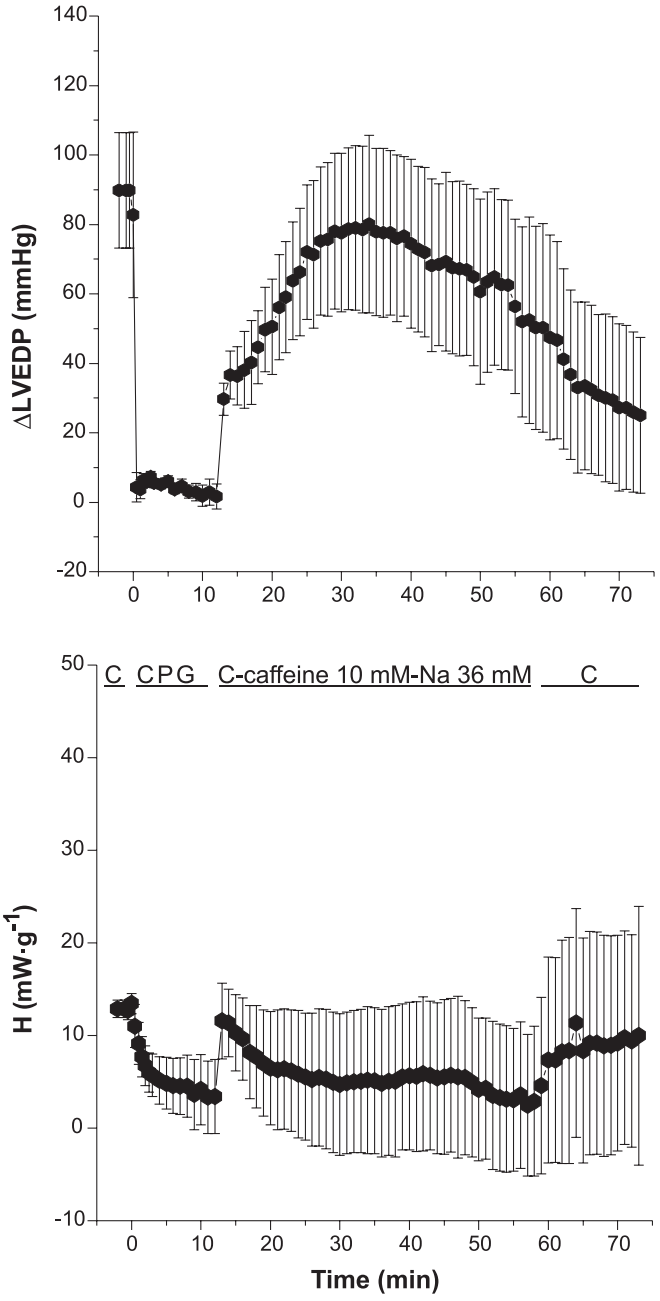


Table 4. Changes in diastolic contracture (ΔLVEDP) and heat release (ΔH_t) produced at the beginning of reperfusion with Krebs – 10 mmol/L caffeine – 36 mmol/L Na (Rep-start) and after 45 min (Rep-end) with respect to the ischemic condition and final changes produced by returning the perfusion from that medium to control-Krebs solution (reversion).

	ΔLVEDP (mmHg)			ΔH_t (mW/g)		
Pre-treatment (n)	Rep-start	Rep-end	Reversion	Rep-start	Rep-end	Reversion
C (4)	73.3±5.6	34.5±11.0	–16.8±7.4	6.5±0.4	6.5±0.6	2.4±0.9
CPG (6)	84.4±14.6	26.9±9.9	–23.5±7.7	4.5±0.5	4.3±0.9	0.8±0.4
C+CLZP (5)	60.1±16.8	–5.2±7.0‡	–21.6±8.5	8.3±0.8*	8.4±1.0*	1.9±0.6
CPG+CLZP (5)	60.1±12.6	18.8±17‡	–20.6±7.6	5.1±1.5†	3.9±1.4†	2.0±0.8
ANOVA	F = 0.78	F = 2.04	F = 0.117	F = 3.57	F = 4.07	F = 1.15
	p = 0.52	p = 0.14	p = 0.94	p = 0.038	p = 0.025	p = 0.35

Note: See the whole protocol in Fig. 5. The columns Rep-start/end show changes respect to the ischemic condition, the columns Reversion shows changes respect to the Rep-end condition. C, Krebs-control solution; C+CLZP, Krebs-control and clonazepam solution; CPG, high K^+ – low Ca^{2+} solution; CPG+CLZP, high K^+ – low Ca^{2+} and conazepam solution. *p < 0.05 vs. C; †p < 0.05 vs. C+CLZP by Tukey's test; ‡NS from 0 by Student's t test.

Fig. 6. Changes in intraventricular pressure (ΔLVEDP, in mmHg, upper) with respect to the pre-treatment and heat release (lower, in mW/g) of hearts treated with CPG for 15 min to stop beating, perfused with Krebs – 10 mmol/L caffeine – 36 mmol/L Na^+ – 2 mmol/L Ca^{2+} (without ischemia) for 45 min, and finally changing the perfusion to control Krebs (n = 4, mean ± SE).



Ca^{2+} to the SR store through the mNCX. Although there may be differences between I/R and hypoxia–reoxygenation (the first includes a deficiency of oxygen among other mechanisms related to the accumulation of metabolites under the absence of perfusion) we can compare our results with those of Griffiths et al. (1998). These authors have shown that clonazepam reduced the increase in $[Ca^{2+}]_m$ during hypoxia in cardiomyocytes and increased it during reoxygenation. According to these authors, hypoxia causes the disruption of the mitochondrial proton gradient by de-energization with the consequent inhibition of the uniporter (Gunter et al. 1994; Bernardi 1999) and the inversion of the mNCX, which was inhibited by clonazepam. The reoxygenation seemed to restore the mitochondrial proton gradient with the consequent reactivation of the Ca^{2+} uptake through the uniporter and Ca^{2+} efflux through the forward mode of the mNCX, which is sensitive to clonazepam (Griffiths et al. 1998). Nevertheless, as previously discussed in our results, clonazepam induced an increase in relaxation of the caffeine-dependent contracture, suggesting an increase in cytosolic Ca^{2+} removal. Since no other transporter could increase the Ca^{2+} removal under C – caff – low Na^+, the results suggest that clonazepam induces a predominance of the Ca^{2+} removal by increasing the gradient for the mitochondrial uniporter upon inhibition of the mNCX. This suggestion is also supported by the measured ΔH_t, which was higher in hearts pretreated with clonazepam under both C and CPG conditions than in the respective pre-treatments without the drug. The higher ΔH_t can be associated to an increase in $[Ca^{2+}]_m$ with the consequent activation of the mitochondrial respiratory chain and heat release, and suggests that clonazepam induces a predominance of the Ca^{2+} uniporter over the mNCX functioning. As it was previously discussed, the ΔH_t remaining over the energetic consume of the other mechanisms (about 4.6 mW/g) could be released by the mitochondria. It is equivalent to about 9.6 nmol $O_2 \cdot g^{-1} \cdot s^{-1}$ (or 13.2 mL $O_2 \cdot kg^{-1}$ min^{-1}), which is much lower than the total O_2 consumption reported for the hearts under steady-state conditions, 100 mL $O_2 \cdot kg^{-1} \cdot min^{-1}$ (Bers 2001).

The role of the mNCX and the uniporter during I/R also explains the reduced contractility when ischemic hearts were reperfused with control Krebs (Fig. 3) solution. The recovery of P during reperfusion, which mostly depends on SR function, was more affected by clonazepam in CPG than in C-hearts, without changes in muscle economy (P/H_t), suggesting that mitochondria contribute Ca^{2+} to the SR store through the mNCX. This result agrees with previous works in which CPG increased both the basal and the active Ca^{2+}- and oxygen-dependent heat fractions associated with mitochondrial activities (Márquez et al. 1997; Consolini et al. 1997). On the other hand, in C hearts, CLZP induced an increase in LVEDP during reperfusion, which suggests a Ca^{2+} accumulation in cytosol. Therefore, an inhibition of the reversal mNCX during ischemia (as it was described for hypoxia) could explain a cytosolic accumulation, which may be increased during reperfusion by 1 or more of the following pathways: impairment of the SR uptake, reversion of mNCX to forward mode by reoxygenation or triggering of the MTP, which is activated by Ca^{2+} overload or depolarization (Di Lisa et al. 2003). In contrast, in cardioplegic hearts, CLZP did not affect the prevention of diastolic contracture during I/R, suggesting that another mechanism would remove the cytosolic Ca^{2+}. In fact, we have previously described that CPG increased the forward SL-NCX activity (Consolini et al. 2004). Also, since CPG increased the basal and active heat fractions associated with mitochondria, it could be possible that CPG avoids the MTP activation or contributes to reduce the reversion of mNCX during ischemia, stimulating the cycling between Ca^{2+} influx by uniporter and Ca^{2+} efflux by mNCX. This cycling could be the contributor to the SR storing during the subsequent reperfu-

sion, as previously discussed. Our results also agree with the reports in which a high-K^+ medium reduced the $[Ca^{2+}]_i$ (Stowe et al. 2000) and retarded the increase in cytosolic $[Ca^{2+}]$ during ischemia (Brachmanski et al. 2004).

In summary, our results on intact contractile hearts support the protective role of mitochondria during reversible I/R. Depending on the state of energization, these organelles could contribute to reduce diastolic contracture by reuptaking Ca^{2+} via the uniporter or the reverse mNCX and release it through the forward mNCX to SR stores. When the mNCX is blocked by clonazepam, diastolic contracture by Ca^{2+} accumulation in cytosol and worsening of contractility recovery during reperfusion occurred. Our results reject the postulated protective effect of clonazepam against I/R (Griffiths et al. 1998; Cox and Matlib 1993) and confirm a deleterious one.

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