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Cardiac basal metabolism: energetic cost of calcium withdrawal in the adult rat heart

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Abstract

Aim: Cardiac basal metabolism upon extracellular calcium removal and its relationship with intracellular sodium and calcium homeostasis was evaluated.

Methods: A mechano-calorimetric technique was used that allowed the simultaneous and continuous measurement of both heat rate and resting pressure in arterially perfused quiescent adult rat hearts. Using pharmacological tools, the possible underlying mechanisms related to sodium and calcium movements were investigated.

Results: Resting heat rate (expressed in mW $g^{-1}_{dry\ wt}$) increased upon calcium withdrawal (+4.4 ± 0.2). This response was: (1) unaffected by the presence of tetrodotoxin (+4.3 ± 0.6), (2) fully blocked by both, the decrease in extracellular sodium concentration and the increase in extracellular magnesium concentration, (3) partially blocked by the presence of either nifedipine (+2.8 ± 0.4), KB-R7943 (KBR; +2.5 ± 0.2), clonazepam (CLO; +3.1 ± 0.3) or EGTA (+1.9 ± 0.3). The steady heat rate under Ca^{2+}-free conditions was partially reduced by the addition of Ru360 (−1.1 ± 0.2) but not CLO in the presence of EGTA, KBR or Ru360.

Conclusion: Energy expenditure for resting state maintenance upon calcium withdrawal depends on the intracellular rise in both sodium and calcium. Our data are consistent with a mitochondrial Ca^{2+} cycling, not detectable under normal calcium diastolic levels. The experimental condition here analysed, partially simulates findings reported under certain pathological situations including heart failure in which mildly increased levels of both diastolic sodium and calcium have also been found. Therefore, under such pathological conditions, hearts should distract chemical energy to fuel processes associated with sodium and calcium handling, making more expensive the maintenance of their functions.

Keywords basal metabolism, calcium depletion, calorimetry, mitochondrial Ca^{2+} cycling.

Energy expenditure by the quiescent myocardium includes the operation of active transport systems associated with the sarcolemma (SL), sarcoplasmic reticulum (SR) and mitochondria (MIT) which maintain ionic cell homeostasis. It has been shown that cardiac resting metabolism is affected by changes in extracellular ionic composition which alter the behaviour of the associated ionic transport systems (Ponce-Hornos *et al.* 1987, 1992, Cooper *et al.* 2001). Although changes in extracellular calcium concentration (Ca^{2+}_o) in the submillimolar to millimolar range do not modify the energetic cost associated with cardiac

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resting state maintenance (Ponce-Hornos & Taquini 1986, Loiselle 1987), there are few reports regarding the energetic consequences of the extracellular calcium withdrawal and the underlying mechanisms involved (Cheung *et al.* 1982, Burkhoff *et al.* 1990, Gibbs & Loiselle 2001).

Calcium removal from the perfusion media abolishes contraction and predisposes cardiac muscle to the so-called 'Ca^{2+} paradox' when calcium is readministered (Zimmerman & Hulsmann 1966). The structural and ionic modifications involved in such a condition have been extensively studied (Ganote & Nayler 1985, Chapman & Tunstall 1987). In this regard, it is well known that Ca^{2+} repletion after a period of nominally free Ca^{2+} perfusion promotes a massive Ca^{2+} entry causing contracture and structural lesions in the myocardium which involve the disruption of the SL at weakened intercalated disc areas leading to loss of cellular constituents and cell death (Grinwald & Nayler 1981, Ganote & Sims 1984). On the other hand, increased membrane permeability to sodium ions has been described upon extracellular Ca^{2+} removal (Bhojani & Chapman 1990, Bosteels *et al.* 1999). It was postulated that sodium ions would enter the cell through L-type calcium channels (Bhojani & Chapman 1990) and/or an unspecific pathway (Mubagwa *et al.* 1997). Several transport mechanisms are linked to intracellular sodium concentration (Na^+_i) handling including the Na^+-K^+ pump, sarcolemmal and mitochondrial Na^+-Ca^{2+} (NCE), Na^+-H^+ (NHE) exchangers, etc. (Bers *et al.* 2003). Therefore, changes in Na^+_i will alter the behaviour of Na^+_i-dependent transport systems which in turn will modify the associated energetic cost. Despite the fact that total cellular calcium decreases during calcium depletion (Cheung *et al.* 1982), a change in cytosolic calcium concentration (Ca^{2+}_i) has remained a controversial issue. Towards this end, Diederichs (1994) reported an increase in Ca^{2+}_i of hearts exposed to nominally Ca^{2+}-free solutions but a decrease was found by others (Auffermann *et al.* 1990, Jansen *et al.* 2000).

The purpose of the present work was to evaluate the energetic consequences of extracellular calcium removal in the quiescent heart muscle. Online calorimetry was employed to this aim because it is a sensitive technique that has been successfully used for studying the energetic of Ca^{2+} movements in the well perfused resting and steadily beating heart (Ponce-Hornos *et al.* 1995, Márquez *et al.* 1997, Bonazzola *et al.* 2002, Consolini & Bonazzola 2008). To avoid a possible Ca^{2+}-dependent cross-bridge cycling upon Ca^{2+}_o withdrawal and its associated contribution to energy expenditure, the cardioplegic agent 2,3-butanedione monoxime (BDM) was used. Thus, this methodological approach allows us to focus on the transport mechanisms associated with Na^+ and/or Ca^{2+} movements. Using selective inhibitors of certain Na^+ and/or Ca^{2+} transporters we evaluated the underlying mechanisms that could be contributing to the observed myothermic response. Our data suggest that not only Na^+_i but also Ca^{2+}_i increase during exposure of hearts to nominally Ca^{2+}-free perfusion. The rise in Na^+_i and Ca^{2+}_i would trigger an enlarged cardiac energy expenditure that seems to be partially associated with a mitochondrial Ca^{2+} cycling which is not detected at physiological levels of extracellular Ca^{2+}. Our findings are potentially relevant in a pathophysiological context because they mimic, at least in part, pathological conditions such as heart failure in which both diastolic Na^+_i and Ca^{2+}_i are mildly increased.

Methods

Biological preparation

Male Wistar rats (280–320 g weight) were heparinized (2000 U) and killed by an intraperitoneal injected thiopental overdose (50–60 mg kg^{-1}). The beating heart was rapidly removed from the animal, cannulated through the aorta by the Langendorff method and perfused at room temperature (20–24 °C). The preparation was mounted in a Kel-F frame for calorimetric measurements. Both atria were carefully dissected from the heart and a small cut in the septal wall close to the aorta was performed in order to prevent spontaneous contractile activity. A water-filled latex balloon was introduced in the left ventricular cavity to measure resting pressure (RP) throughout the experiment. After cannulation and mounting the heart was placed in the inner chamber of a calorimeter (Ponce-Hornos *et al.* 1982, 1995) and the latex balloon was connected to a Statham P23db pressure transducer (Goold-Statham Instruments, Hato Rey, Puerto Rico). All experiments were carried out at 25 °C. At the end of each experiment the tissue was removed from the calorimeter, weighed in a pre-weighed vial, and dried at 110 °C to constant weight so that water content could be calculated. The averaged water content in the present experiments was 82.9 ± 0.2% (n = 110).

All animal procedures and experimental protocols were approved by the Ethics Committee for Research of the School of Medicine of the Buenos Aires University and conform to the *Guide for the Care and Use of Laboratory Animals NIH publication no. 85-23*, revised 1996.

Solutions and protocols

The hearts were perfused at a constant rate (5 mL min^{-1}) with a solution (control solution) containing (in mmol L^{-1}): NaCl 135, $CaCl_2$ 1.0, $MgCl_2$

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0.3, KCl 3.6, dextrose 16, BDM 10, Tris 5. After calorimetric equilibration of the preparation (approx. 1.5 h), perfusion was switched to a solution of similar composition as the control solution without $CaCl_2$ added ($0Ca^{2+}$) until steady state heat production was achieved. In some experiments, before exposing the tissue to the $0Ca^{2+}$ medium, either extracellular sodium concentration (Na^+_o) was decreased to 40 mmol L^{-1} [osmolarity was compensated with choline chloride in the presence of atropine (1 mg L^{-1})] or extracellular magnesium concentration (Mg^{2+}_o) was increased to 3 mmol L^{-1}. Afterwards, under $0Ca^{2+}$ perfusion Na^+_o was increased to 135 mmol L^{-1} or Mg^{2+}_o was returned to its control value. All solutions were neutralized to pH 7.4 and continuously bubbled with 100% O_2. Nifedipine (NIF, 3 μmol L^{-1}), tetrodotoxin (TTX, 10 μmol L^{-1}), KB-R7943 (KBR, 5 μmol L^{-1}) clonazepam (CLO, 10 μmol L^{-1}), Ru360 (1 μmol L^{-1}) or EGTA (10 μmol L^{-1}) were added to the perfusion solution to evaluate their effect on the myothermic response to Ca^{2+} removal. All drugs were added to the perfusion media from stock solutions in dimethyl sulfoxide except for EGTA and Ru360 which were added from stock aqueous solutions. NIF, TTX, EGTA and CLO were purchased from Sigma-Aldrich (Buenos Aires, Argentina), KBR was from Tocris Cookson (Ellisville, MO, USA) and Ru360 was obtained from Calbiochem (EMD Chemicals, Sibbstown, NJ, USA).

Heat and mechanical measurements

The technique for measurement of heat production and mechanical activity of the isolated heart has been described previously in detail (Ponce-Hornos *et al.* 1982, 1995). Briefly, the calorimeter was submerged in a constant temperature bath. The temperature of the calorimeter bath (25 °C) was controlled with a cooling-heating bath (±0.003 °C) in which the perfusate was also equilibrated. The calorimeter used two thermoelectric modules [two isolated ceramic modules (Melcor Thermoelectrics, Trenton, NJ, USA) with 127 thermosensitive junctions each]. The response of the calorimeter to maintained heat liberation follows a diffusion-type function that considers that the heat released approaches equilibrium exponentially at the peltier units (Ponce-Hornos *et al.* 1995). The rate constant was about 0.03 s^{-1} under the perfusion conditions used in the present experiments. Calorimeter calibration was accomplished by passing a 2.1 kHz sine wave through the muscle by means of stimulating electrodes in the inner chamber of the calorimeter (Ponce-Hornos *et al.* 1982). With this method it was possible to continuously and simultaneously record RP, perfusion pressure and heat rate of the biological preparation. During the equilibration period inside the calorimeter and before exposure to BDM-containing perfusion, the electrically stimulated beating heart (0.5 Hz) was stretched by steps (~2 mmHg each) until maximal developed pressure was reached. RP under optimal length ranged between 8 and 16 mmHg. Hearts were accepted for analysis if the developed pressure was at least sixfold of its respective RP. Afterwards the hearts were kept at rest by either stopping electrical stimulation (in some experiments) or by exposure to the BDM-containing solution without electrical stimulation. After that RP increased in variable degree, at first rapidly and later more slowly, until a new steady state was reached. Thus, the RP of the quiescent heart under control conditions ranged between 10 and 25 mmHg. The RP and heat rate outputs were either recorded in a Grass (model 7; Braintree, MA, USA) Polygraph recorder or digitalized and stored with an A/D converter (TL-1 DMA; Axon Instruments, Foster City, CA, USA) in an AT 486 desk computer. A sampling frequency of 1 sample per second was used.

Statistical analyses

Data are presented as mean ± standard error of the mean (SEM), where n represents the number of hearts analysed. Statistical significance was settled at the $P < 0.05$ level. For paired comparisons a paired t-test was used. When more than two experimental groups were compared ANOVA was used instead. When ANOVA was significant, the Duncan multiple rank test was used.

Results

After an equilibration period of 1.5 h under control perfusate, the hearts were exposed to a nominally Ca^{2+}-free solution ($0Ca^{2+}$). RP rose during the first 25–30 min of exposure to $0Ca^{2+}$ and remained stable thereafter (Fig. 1b). As there were no significant differences among the changes observed for the different series of experiments where Ca^{2+} removal was done, data were pooled and averaged. The average RP change was 6.2 ± 0.3 mmHg (n = 40). The different experimental conditions tested after Ca^{2+} removal did not induce subsequent significant changes in RP. Resting heat rate (H_r) under control conditions for all 110 experiments performed was 26.4 ± 0.1 mW $g^{-1}_{dry\ wt}$. As shown in Figure 1a, Ca^{2+} withdrawal monotonically increased H_r reaching a new steady state after approx. 30–35 min in $0Ca^{2+}$. In some experiments there was an initial transitory fall in H_r during the first 3–5 min under $0Ca^{2+}$ which was reversed thereafter. The average increase in H_r was +4.4 ± 0.2 mW $g^{-1}_{dry\ wt}$ ($P < 0.05$) for the 40 experiments where extracellular Ca^{2+} was removed. The source of such a myothermic response could be related to the reported increase in cellular membrane permeability to monovalent cations such as

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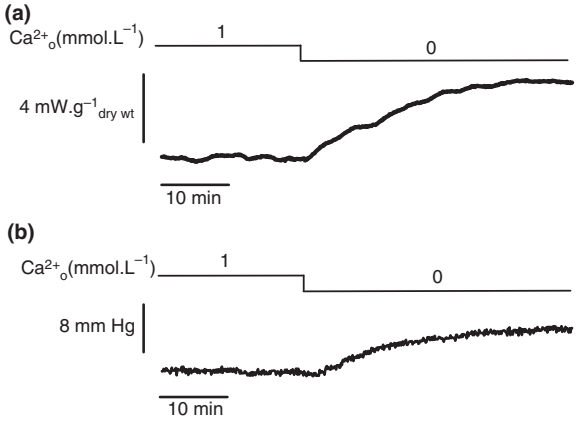

Figure 1 Effect of extracellular calcium removal on the myothermal and mechanical responses in the quiescent rat heart. Original digitalized records for resting heat rate (a) and resting pressure (b). At the top of each panel the change in extracellular calcium concentration (Ca^{2+}_o) is indicated.

sodium ions (Bhojani & Chapman 1990, Bosteels *et al.* 1999). Therefore, Na^+_o was decreased in the Ca^{2+}-containing perfusate to 40 mmol L^{-1} before exposing the hearts to $0Ca^{2+}$ medium. Figure 2 shows the myothermic response to $0Ca^{2+}$ exposure when transarcolemmal sodium gradient was previously decreased. Neither the decrease in Na^+_o in the presence of Ca^{2+} nor the subsequent Ca^{2+} withdrawal induced any significant changes in H_r. The return to control Na^+_o (135 mmol L^{-1}) again increased H_r to the level observed in $0Ca^{2+}$ perfusion (Fig. 2). The average increase in H_r by sodium increase was +4.3 ± 0.3 mW $g^{-1}_{dry\ wt}$ ($P < 0.05$, $n = 10$).

The myothermic response to $0Ca^{2+}$ perfusion was not modified by the presence of the Na^+ channel blocker TTX (10 μmol L^{-1}) but was significantly decreased ($P < 0.05$) by the presence of the Ca^{2+} channel blocker NIF (3 μmol L^{-1}) (Fig. 3). Each drug was introduced in the perfusion medium before calcium removal and remained in the perfusate thereafter. Neither TTX nor NIF induced any significant changes in H_r under normal Ca^{2+} perfusion (−0.1 ± 0.2 and −0.2 ± 0.2 mW $g^{-1}_{dry\ wt}$ for TTX and NIF respectively). The average increase in H_r upon Ca^{2+} removal was +4.3 ± 0.6 mW $g^{-1}_{dry\ wt}$ ($n = 6$) and +2.8 ± 0.4 mW $g^{-1}_{dry\ wt}$ ($n = 11$) for TTX and NIF respectively (Fig. 3).

On the other hand, the myothermic response to Ca^{2+} removal was fully abolished by an increased extracellular Mg^{2+} concentration (Mg^{2+}_o, 3 mmol L^{-1}) (Fig. 4). Conversely, under $0Ca^{2+}$ perfusion, the subsequent decrease in Mg^{2+}_o elicited an increase in H_r (Fig. 4a) whose value amounted to +4.0 ± 0.4 mW $g^{-1}_{dry\ wt}$ ($n = 8$, Fig. 4b).

The results obtained so far suggest that the rise in H_r induced by extracellular Ca^{2+} removal is associated

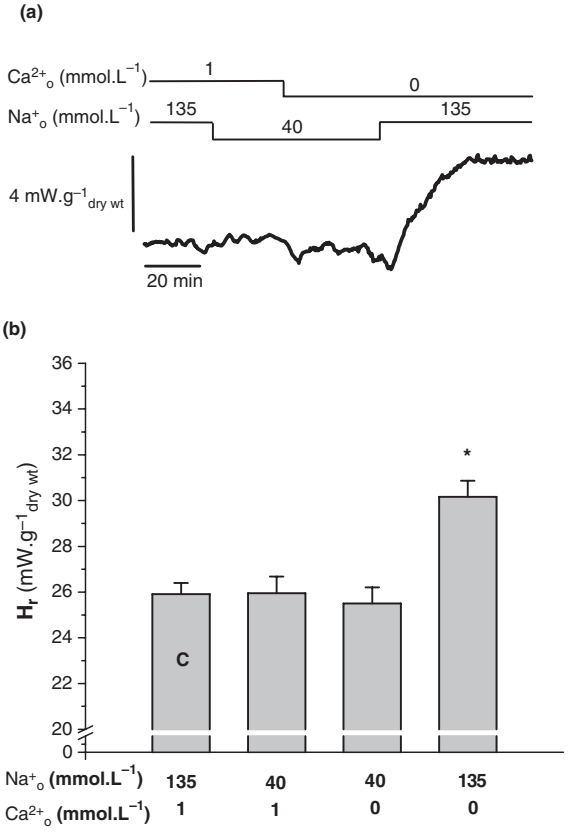

Figure 2 Effect of the extracellular sodium concentration (Na^+_o) decrease on the myothermic response to Ca^{2+} withdrawal. (a) Representative record of resting heat rate (H_r) after the fall in Na^+_o in the presence followed by the absence of extracellular Ca^{2+}(Ca^{2+}_o). Sodium increase in the absence of Ca^{2+}_o is also shown. Horizontal lines at the top of the figure indicate the changes in extracellular ionic composition. (b) Summarized data (mean ± SEM) from 10 experiments of the type shown in (a). C, control condition; *$P < 0.05$ vs. C.

with a Na^+ influx to the myocardial cell through pathways other than TTX-sensitive Na^+ channels. Because Ca^{2+}_o in our Ca^{2+}-free solutions was not zero (typically 2–3 μmol L^{-1} as measured electrochemically), it could be possible that the observed myothermic response could partly be linked to Ca^{2+}, as the results with NIF suggest. Due to the fact that the increase in Na^+_i could induce activation of Na^+_i-dependent processes such as the reverse mode of the sarcolemmal Na^+-Ca^{2+} exchanger (SL-NCE) and Ca^{2+} mobilization at the mitochondrial level, we tested if those processes could be contributing to the observed change in basal metabolism induced by $0Ca^{2+}$ exposure.

To explore the possibility that the increased Na^+_i affects Ca^{2+} movement through the inner mitochondrial membrane, the mitochondrial Na^+-Ca^{2+} exchanger (mit-NCE) inhibitor CLO (10 μmol L^{-1}) was used (Cox & Matlib 1993). After steady heat rate was

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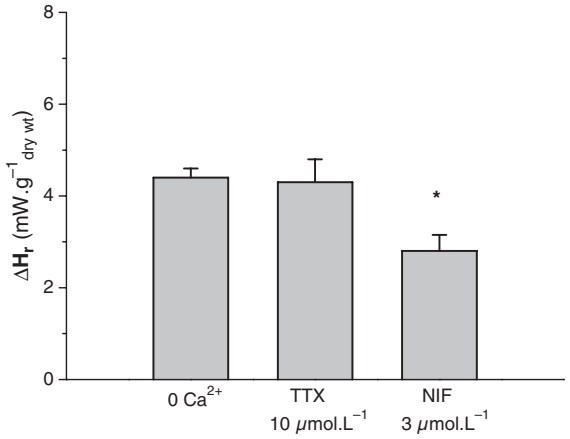

Figure 3 Effects of Na^+ and Ca^{2+} channel blockers on resting heat rate response to Ca^{2+} removal. ΔH_r is the change in resting heat rate in the presence of the Na^+ channel blocker tetrodotoxin (TTX) 10 μmol L^{-1} (n = 6 experiments) or the Ca^{2+} channel blocker nifedipine (NIF) 3 μmol L^{-1} (n = 11 experiments). Both drugs were present before Ca^{2+} withdrawal and remained in the perfusate thereafter. H_r change upon Ca^{2+} removal without any drugs present ($0Ca^{2+}$) was included for comparison (n = 40 experiments). Bars represent mean SEM. *P < 0.05 vs. $0Ca^{2+}$.

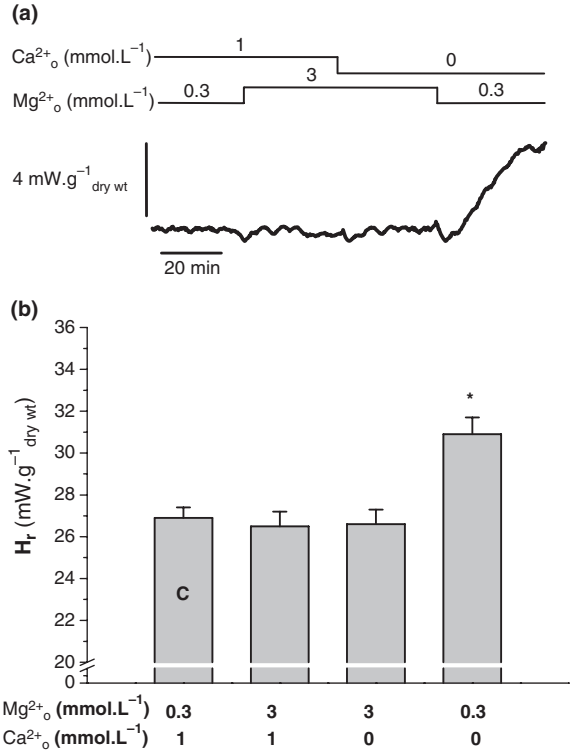

Figure 4 Effect of extracellular Mg^{2+} concentration (Mg^{2+}_o) on resting heat rate (H_r) response to Ca^{2+} withdrawal. (a) Representative record of H_r before and after the increase in Mg^{2+}_o in the presence of normal extracellular Ca^{2+} concentration (Ca^{2+}_o) followed by Ca^{2+} removal, as indicated at the top of the panel. H_r response to Mg^{2+}_o decrease under Ca^{2+}-free medium is also shown. (b) Summarized data for H_r from eight experiments like that shown in (a). Bars represent mean ± SEM. C, control condition. *P < 0.05 vs. C.

achieved under $0Ca^{2+}$ perfusion, introduction of CLO to the perfusion medium (Fig. 5a,b) significantly decreased H_r (-1.3 ± 0.2 mW g^{-1} $_{dry\ wt}$, n = 8, P < 0.05). In another group of experiments CLO was present before Ca^{2+} removal (Fig. 5c,d). In the presence of calcium the drug did not produce any significant change in H_r. Ca^{2+} withdrawal in the presence of CLO induced an increase in basal metabolism ($+3.1 \pm 0.3$ mW g^{-1} $_{dry\ wt}$, n = 12) that was significantly less (P < 0.05) than that observed in its absence, suggesting that the mit-NCE is active under $0Ca^{2+}$ perfusion and that cytosolic Ca^{2+} may be increased under such conditions. To further evaluate this possibility, hearts were exposed to the $0Ca^{2+}$ perfusate in the presence of the Ca^{2+} chelator EGTA (10 μmol L^{-1}). As shown in Figure 6, H_r rose to a new steady state level that was significantly (P < 0.05) less than that observed without EGTA. The average change in cardiac basal metabolism in the presence of EGTA was 1.9 ± 0.3 mW g^{-1} $_{dry\ wt}$ (n = 11). Furthermore, the subsequent addition of CLO (10 μmol L^{-1}) did not modify H_r (Fig. 6a,b). These results strongly suggest that not only Na^+_i but also Ca^{2+}_i are increased by exposure to nominally Ca^{2+}-free solution. About 60% of the myothermic response to $0Ca^{2+}$ can be ascribed to a rise in Ca^{2+}_i. The lack of a CLO effect in the presence of EGTA suggests that the mit-NCE does not play a significant role when Ca^{2+}_i remains low as opposed to its effect in the presence of a $0Ca^{2+}$ solution.

The Ca^{2+}-dependent effect of CLO could be due to either an activation of the reverse mode of mit-NCE or a Ca^{2+} cycling at the inner mitochondrial membrane which is triggered by exposure to $0Ca^{2+}$, hence leading to higher energy expenditure. To explore the possibility that mitochondrial Ca^{2+} cycling is active under $0Ca^{2+}$ conditions, the hearts were treated with the selective inhibitor of mitochondrial calcium uniporter (mit-CaU) Ru360 (1 μmol L^{-1}) (Matlib *et al.* 1998) followed by the exposure to CLO (10 μmol L^{-1}) after steady H_r was achieved in $0Ca^{2+}$. As shown in Figure 7, Ru360 decreased H_r (-1.1 ± 0.2 mW g^{-1} $_{dry\ wt}$, n = 10) and the subsequent exposure to CLO had no significant effect on it, suggesting that mitochondrial cycling contributes to the increased energy expenditure induced by extracellular Ca^{2+} removal.

On the other hand, the presence of contaminating Ca^{2+} in the perfusate together with increased Na^+_i, plus the fact that cells depolarize upon $0Ca^{2+}$ exposure (Diederichs 1994, Mubagwa *et al.* 1997), could favour a possible

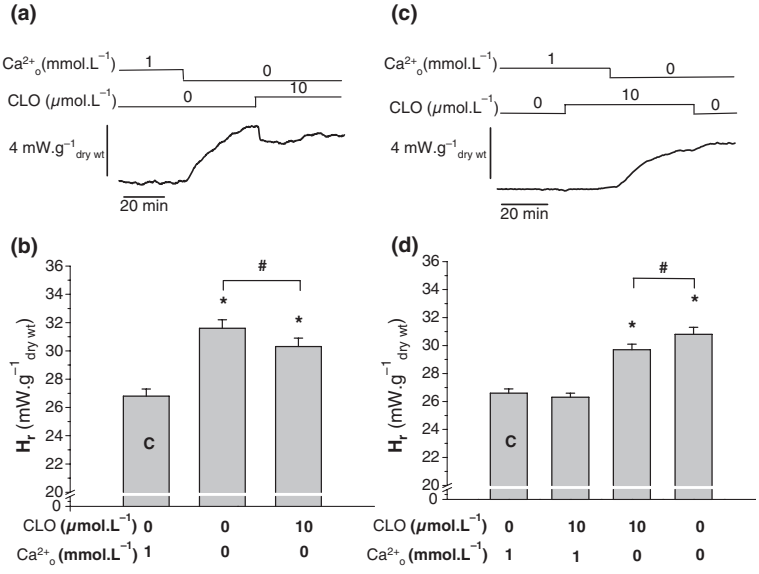

Figure 5 Effect of clonazepam (CLO) on resting myothermic response (H_r) to Ca^{2+} removal. (a) Representative record of H_r before and after Ca^{2+} withdrawal followed by the addition of CLO (10 μmol L^{-1}) to the perfusate as indicated at the top of the panel. (b) Summarized data for H_r from eight experiments like that shown in panel (a). (c) Representative record of H_r when CLO (10 μmol L^{-1}) was present before Ca^{2+} removal as indicated at the top of the panel. CLO washout is also shown. (d) Summarized data for H_r from 12 experiments like that depicted in panel (c). Bars in (b) and (d) represent mean ± SEM. C, control condition. $^*P < 0.05$ vs. C. $^{\#}P < 0.05$ between indicated bars.

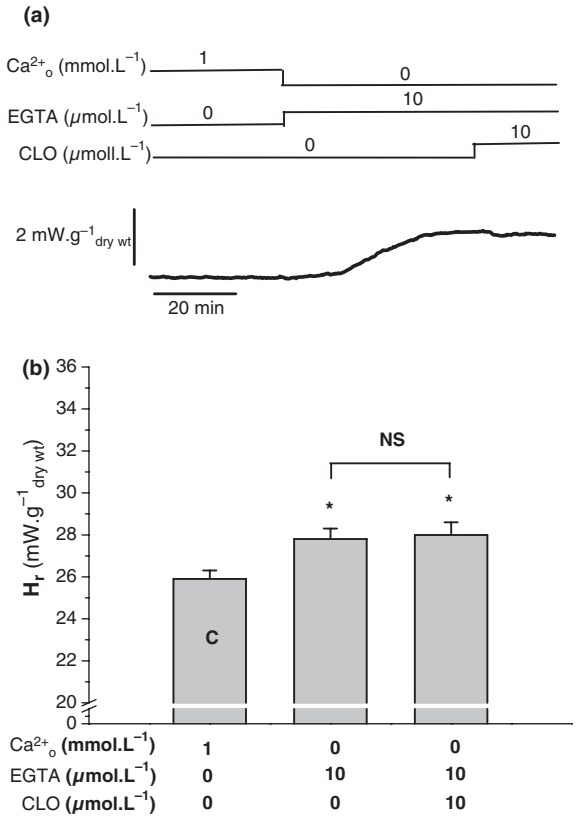

Figure 6 Effect of EGTA on cardiac basal metabolism under Ca^{2+}-free perfusion. (a) Representative record of resting heat rate (H_r) after exposure to Ca^{2+}-free medium in which EGTA (10 μmol L^{-1}) was present, followed by the addition of clonazepam (CLO) 10 μmol L^{-1} as indicated at the top of the panel. (b) Summarized data for H_r from 11 experiments as that depicted in panel (a). Bars represent mean ± SEM. C, control condition. $^*P < 0.05$ vs. C. NS, non-significant difference between indicated bars.

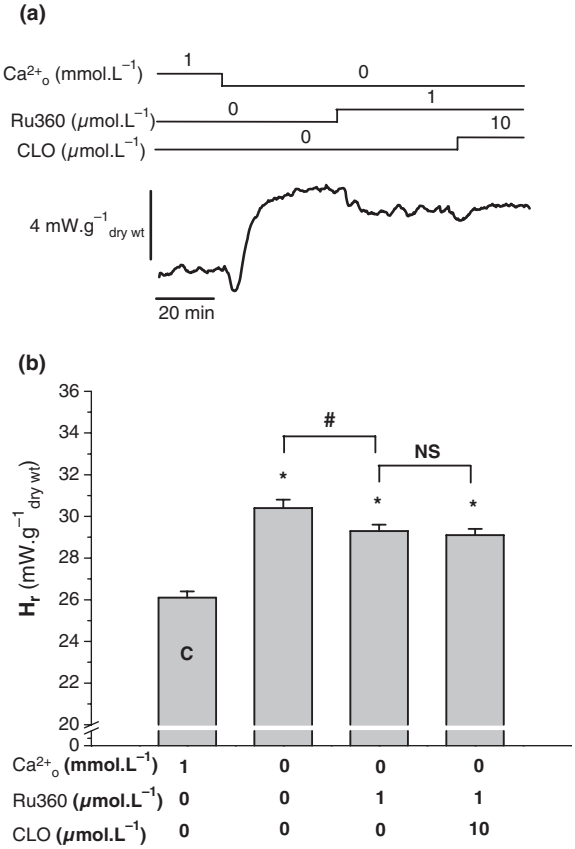

Figure 7 Effect of Ru360 on resting heat rate (H_r) in Ca^{2+}-free medium. (a) Representative record of H_r after Ca^{2+} withdrawal followed by the sequential addition of Ru360 (1 μmol L^{-1}) and CLO (10 μmol L^{-1}) to the perfusate as indicated at the top of the panel. (b) Summarized data for H_r from 10 experiments like that shown in panel (a). Bars represent mean ± SEM. C, control condition. $^*P < 0.05$ vs. C. $^{\#}P < 0.05$. NS, non-significant difference between indicated bars.

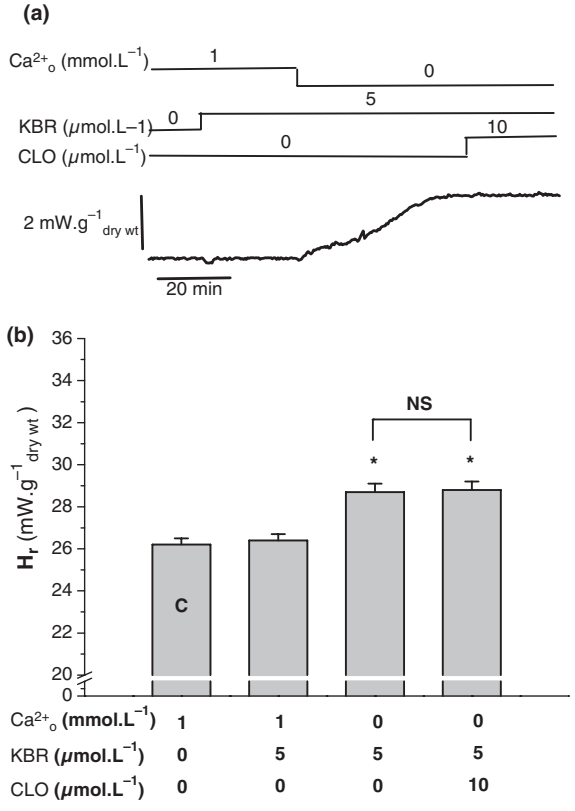

Figure 8 Effect of KB-R7943 (KBR) on resting heat rate (H_r) response to Ca^{2+} withdrawal. (a) Representative record of H_r when KBR (5 μmol L^{-1}) was present before Ca^{2+} removal with the subsequent addition of clonazepam (CLO) 10 μmol L^{-1} in Ca^{2+}-free perfusion as indicated at the top of the panel. (b) Summarized data for H_r from 12 experiments like that depicted in panel (a). Bars represent mean ± SEM. C, control condition. *$P < 0.05$ vs. C. NS, non-significant difference between indicated bars.

calcium influx to the myocytes through the reverse mode of SL-NCE. Therefore, the $0Ca^{2+}$ intervention was carried out in the presence of the reverse mode SL-NCE inhibitor KBR (5 μmol L^{-1}) (Satoh *et al.* 2000). As observed previously (Bonazzola *et al.* 2002), no significant change in H_r was induced by the presence of KBR under control conditions (Fig. 8). The subsequent Ca^{2+} removal produced an increase in H_r (+2.5 ± 0.2 mW g^{-1} $_{dry\ wt}$, $n = 12$) that was significantly less ($P < 0.05$) than that observed in the absence of the drug. This suggests the possibility of a contribution of Ca^{2+} influx through the SL-NCE working in its reverse mode to the energetic response to extracellular Ca^{2+} withdrawal. Once again H_r was not changed by the subsequent addition of CLO (10 μmol L^{-1}) to the perfusate (Fig. 8).

Discussion

In this work we studied the changes in cardiac basal metabolism [evaluated as resting heat rate (H_r)] in response to exposure to a nominally Ca^{2+}-free medium ($0Ca^{2+}$). The main findings include the following: (1) a rise in basal metabolism that relates not only to the already reported increase in Na^+_i (Bhojani & Chapman 1990, Jansen *et al.* 1998, Bosteels *et al.* 1999) but also to an increased Ca^{2+}_i; (2) the changes in heat rate fraction associated with Ca^{2+}-dependent transport processes were quantitatively the most important ones; (3) both mit-CaU and mit-NCE are involved in the myothermic response to extracellular Ca^{2+} withdrawal; and (4) the altered cellular ionic homeostasis would trigger a mitochondrial Ca^{2+} cycling that contributes to the energetically more expensive basal metabolism maintenance under $0Ca^{2+}$.

Although the cardioplegic agent BDM was present in the perfusate, cardiac resting heat rate measured under control conditions (4.5 mW g^{-1}) was similar to those previously reported by us and other investigators (4.09–4.78 mW g^{-1}) in adult rat hearts (Gibbs & Loiselle 1978, Bonazzola *et al.* 1992, Ponce-Hornos *et al.* 1995, Loiselle *et al.* 1996, Márquez *et al.* 1997). In fact, in some experiments, exposure to BDM (10 mmol L^{-1}, data not shown) was without significant change on H_r as was observed by others (Schramm *et al.* 1994). Extracellular Ca^{2+} removal raised H_r to a new steady state level. The magnitude of the measured H_r change (+4.4 ± 0.2 mW g^{-1} $_{dry\ wt}$) was less than that previously observed (+6.3 ± 0.5 mW g^{-1} $_{dry\ wt}$) by exposure to high K^+ (24 mmol L^{-1}) cardioplegia (Ponce-Hornos *et al.* 1992). This fact is in agreement with Burkhoff *et al.* (1990) who reported that the way in which cardiac quiescence was achieved determines the energetic cost of resting state maintenance.

Calcium removal induced a significant increase in RP (6.2 mmHg, Fig. 1) that remained stable after 25–30 min of exposure to $0Ca^{2+}$. In the present experiments, the presence of BDM in the perfusate should preclude the possibility of a Ca^{2+}-dependent contracture and its associated rise in energy expenditure. As the presence of an important oedema was commonly found, it should be the reason for the observed RP increase and consequently RP rise would not contribute to the myothermal response under $0Ca^{2+}$.

The increased myothermal response to Ca^{2+}_o withdrawal may reflect the activation of active transport mechanisms that would counteract the altered ionic homeostasis induced by $0Ca^{2+}$. It is known that Ca^{2+}-free perfusion elicits Na^+ influx into cardiomyocytes (Bhojani & Chapman 1990, Jansen *et al.* 1998, Bosteels *et al.* 1999). Our experiments in which a decrease in Na^+_o before Ca^{2+} removal was imposed and mainly the subsequent Na^+ increase under $0Ca^{2+}$ (Fig. 2) are in agreement with such previous observations. Nevertheless, no change in heat rate was observed upon reduction of Na^+_o to 40 mmol L^{-1} in the presence of

calcium (see Fig. 2) while Cooper *et al.* (2001), working on rat hearts rendered quiescent with high-K^+ cardioplegia at 37 °C, reported a dramatic increase in oxygen consumption. Under control conditions, the SL-NCE should be expected to function in its forward mode (i.e. Na^+_o-dependent Ca^{2+}_i efflux) and the calculated driving force (DF) for the exchanger shows that this is, indeed, the case. Thus, for the Na^+_o (135 mmol L^{-1}) and Ca^{2+}_o (1 mmol L^{-1}) present in our control perfusate and assuming a normal diastolic Ca^{2+}_i (0.1 μmol L^{-1}) and Na^+_i (11.1 mmol L^{-1}; Despa *et al.* 2002), and a resting potential of about −80 mV, at the working temperature (25 °C) the DF (mV) for the SL-NCE can be calculated according to the formula $DF = nE_{Na} - 2E_{Ca} - E_m$ $(n - 2)$, where E_{ion} is the equilibrium potential, E_m is the membrane potential and coupling ratio $n = 3$ whose value amounts to +39 mV. Thus, the resulting positive DF sign indicates that under initial control conditions the Ca^{2+} outward transport prevails. Conversely, when Na^+_o is decreased to 40 mmol L^{-1}, the calculated DF reaches −58 mV indicating that the SL-NCE would be operating in its reverse mode causing Na^+_i-dependent Ca^{2+}_o influx and therefore an increase in heat rate would be expected. The fact that no significant change in H_r was observed upon reduction of Na^+_o in the present experiments can be attributed to the presence of the cardioplegic agent BDM in the perfusion media. To support this contention, it has been reported that BDM inhibits the exchanger (about 80%) at the dose used in the present work (10 mmol L^{-1}) (Watanabe *et al.* 2001, 2006). In fact, in some experiments (data not shown), the reduction of Na^+_o was carried out in the absence and in the presence of BDM, and the expected increase in heat rate was observed only in the absence of the drug. The fact that the presence of TTX does not modify the myothermic response to Ca^{2+}_o removal (Fig. 3) is in agreement with the reported Ca^{2+}_o depletion-induced depolarization and the resulting voltage inactivation of the voltage-dependent Na^+ channels (Mubagwa *et al.* 1997). Also, the results of the effects of NIF and EGTA on the myothermal response to Ca^{2+} removal (Figs 3 and 6) in the quiescent rat heart are both compatible with Na^+ entry mediated by L-type Ca^{2+} channels and/or other unspecific pathways as described previously (Bhojani & Chapman 1990, Bosteels *et al.* 1999). It can be argued that the activation of the forward mode of SL-NCE may be responsible, at least in part, for the Na^+ influx upon removal of Ca^{2+}_o. In fact, if the DF for SL-NCE is calculated (with: Na^+_o = 135 mmol L^{-1}, Ca^{2+}_o = 2.5 μmol L^{-1}, Na^+_i = 11.1 mmol L^{-1}, Ca^{2+}_i = 0.1 μmol L^{-1} and E_m = −80 mV), its value amounts to +133 mV, indicating that the forward mode of the exchanger would be favoured (compare with DF under control conditions, +39 mV, see above). Because the presence of BDM in the perfusate would inhibit both modes of operation of the exchanger (Watanabe *et al.* 2001), such a possibility may be ruled out. It should be noted that in our $0Ca^{2+}$ solutions contaminant Ca^{2+} levels (2–3 μmol L^{-1}) were present, so that a Ca^{2+} influx through L-type Ca^{2+} channels during Ca^{2+}-free perfusion cannot be ruled out (Hess *et al.* 1986). Consequently, the diminished H_r change observed in the presence of NIF (Fig. 3) could include the inhibition of both Na^+ and Ca^{2+} influx. In addition, the null myothermic effect upon Ca^{2+}_o withdrawal observed in the presence of elevated Mg^{2+}_o (Fig. 4) can be attributed to the well-known stabilizing effect of extracellular Mg^{2+} on biological membranes (Reynolds 1972), which includes its ability to exclude monovalent cations from a binding site within L-type Ca^{2+} channels whose occupation is a prerequisite for ion permeation (Hess *et al.* 1986).

Early reports have shown a decrease in total Ca^{2+} content under Ca^{2+}-free perfusion conditions (Borgers & Van Belle 1985). However, measurements of cytosolic calcium concentration in cardiac tissue have rendered controversial results. Thus, using the same species as in the present study, Jansen *et al.* (2000) found a decrease in Ca^{2+}_i assessed fluorometrically during short periods of exposure to Ca^{2+}-depleted solutions, whereas Diederichs (1994) using the ion selective microelectrode technique (ISME) during long periods of exposure reported the opposite. Our EGTA experiments (Fig. 6) clearly suggest that both Na^+ and Ca^{2+} increase within the myocardial cell during $0Ca^{2+}$ perfusion. The Ca^{2+}-dependent component accounts for a main fraction (~60%) of the measured total H_r change induced by $0Ca^{2+}$ exposure. The Na^+-dependent fraction of the energy expenditure under $0Ca^{2+}$ evaluated in the EGTA experiments (1.9 mW $g^{-1}_{dry\ wt}$ or 0.32 mW g^{-1}) could be attributed to the Na^+_i-dependent increased activity of the Na^+-K^+ pump which is increased at higher Na^+_i (Despa *et al.* 2002). Taking into account that, in steady state conditions, the ATP used by the pump will be resynthesized by oxidative phosphorylation rendering 50 kJ mol^{-1} of resynthesized ATP (Gibbs & Loiselle 2001), the stoichiometry of the pump (3 Na^+ transported per ATP hydrolysed) and tissue density of 1 g mL^{-1}, an increase in Na^+ pump-mediated Na^+ efflux of 1.15 mmol Na^+ L^{-1} min^{-1} can be calculated. This value represents approx. 36% of the maximum obtainable rise in Na^+ pump-mediated Na^+ efflux (~3.2 mmol Na^+ L^{-1} min^{-1}) reported by Despa *et al.* (2002) in rat ventricular myocytes at room temperature, and predicts a change in Na^+_i of approx. 2 mmol L^{-1} above the normal resting Na^+_i of rat ventricular cells (about 11.1 mmol L^{-1}; Despa *et al.* 2002). This calculated modest Na^+_i increase is slightly less than that estimated by Bosteels *et al.* (1999) from their Na^+ flourometric measurements on rat ventricular

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myocytes in the absence of both Ca^{2+}_o and Mg^{2+}_o (2.8–5.8 mmol Na^+ L^{-1}), but very far from those reported in ferret ventricular trabeculae by Bhojani & Chapman (1990) (~30 mmol Na^+ L^{-1}) and by Diederichs (1994) in rat ventricle using ISME (~40 mmol Na^+ L^{-1}). The discrepancy could be due to different temperatures used and its profound effects on active transport process capacity. Bhojani & Chapman (1990) and Diederichs (1994) carried out their measurements at 32 and 37 °C, respectively, whereas Bosteels *et al.* (1999) and our estimations were calculated from experiments performed at low temperature (22–25 °C), a temperature similar to that used by Despa *et al.* (2002) for their Na^+ pump-mediated Na^+ efflux measurements.

Among the Na^+_i-dependent processes that could probably contribute to the change in cardiac basal metabolism under Ca^{2+}-free perfusion, a Na^+_i-induced Ca^{2+} mobilization at the mitochondrial level could be playing a role. It is well known that the mitochondrial inner membrane possesses several Ca^{2+} transport systems for the regulation of mitochondrial Ca^{2+}(Ca^{2+}_m) (Bernardi 1999, Gunter *et al.* 2000, O'Rourke 2007). Ca^{2+} enters the MIT via the mit-CaU which is driven by the negative mitochondrial membrane potential supported by mitochondrial respiration. Mitochondrial Ca^{2+} efflux is mediated primarily via mit-NCE in cardiac myocytes and intramitochondrial Na^+ is extruded in exchange with extramitochondrial H^+ through mit-NHE consuming proton motive force. Thus, a Ca^{2+} cycling can be established across the inner mitochondrial membrane that requires energy for its maintenance. The experiments depicted in Figure 5 in which the mit-NCE inhibitor CLO was used (Cox & Matlib 1993) indicate that such a transport system is involved in the energetic response upon Ca^{2+} withdrawal. Because of Na^+_i rise under $0Ca^{2+}$ perfusion, a Na^+_i-dependent activation of the mit-NCE that would drive to a Ca^{2+}_m depletion with the consequent fall of Ca^{2+}_m-activated metabolic rate was expected. Nevertheless, the addition of CLO to the $0Ca^{2+}$ medium induced a decrease rather than the expected increase in the energy released. This opposite result can be explained if either there is a Ca^{2+} influx through mit-NCE acting in its reverse mode (Griffiths *et al.* 1998, Kim & Matsuoka 2008) or alternatively, an activated Ca^{2+} cycling across the inner mitochondrial membrane under Ca^{2+}-free perfusion exists. The results from experiments in which the mit-CaU specific inhibitor Ru360 was used (Fig. 7) agree with the last hypothesis. Thus, when mit-CaU is blocked in $0Ca^{2+}$, the metabolic rate decreases due to the interruption of the postulated Ca^{2+} cycling and, as expected, the subsequent CLO addition has no effect. Furthermore, CLO has no effect on metabolism at low Ca^{2+}_i such as that found in either the quiescent state with normal Ca^{2+}_o (Fig. 5d) or when the Ca^{2+} chelator EGTA was present under Ca^{2+}-free perfusion (Fig. 6), indicating a non-detectable mit-CaU activity at diastolic Ca^{2+}_i levels. It is instructive to estimate how much calcium would be cycling across the inner mitochondrial membrane and hence which would be the associated change in Ca^{2+}_i above the normal resting level. Taking into account that during Ca^{2+} cycling 3 H^+ ions enter the matrix per each Ca^{2+} cycled that must be extruded by the respiratory chain, and assuming that 12 H^+ are extruded per each O_2 reduced, using 1 nmol O_2 g^{-1} s^{-1} = 0.47 mW g^{-1} as the thermal equivalence for O_2 consumption (Gibbs & Loiselle 2001), the change in heat rate measured in the presence of Ru360 (1.1 mW $g^{-1}_{\ dry\ wt}$ or 0.188 mW g^{-1}) is equivalent to a change in Ca^{2+} flux of 1.6 nmol Ca^{2+} g^{-1} s^{-1}. Furthermore, using the reported kinetic parameters (K_m = 15 μmol L^{-1}, V_{max} = 0.5 nmol Ca^{2+} mg^{-1}_{prot} s^{-1}) for mit-CaU in the presence of Mg^{2+} (Carafoli 1985) and that there is about 114 mg_{prot} per gram wet tissue (Crompton 1990), it can be calculated that such increased Ca^{2+} flux corresponds to a Ca^{2+}_i of 433 nmol L^{-1}. This Ca^{2+}_i level is above the threshold (~300 nmol L^{-1}) where Ca^{2+}_m shows a steep dependence on Ca^{2+}_i (Adrianenko *et al.* 2009). The relative increase (taking 100 nmol L^{-1} as normal diastolic Ca^{2+}_i for the rat; Jansen *et al.* 2000) amounts to 3.3 and compares with the twofold increase measured in rabbit ventricular myocytes of hearts who suffer heart failure (Baartscheer *et al.* 2003).

Due to the presence of contaminant Ca^{2+}_o plus the fact that cells depolarize upon $0Ca^{2+}$ exposure (Diederichs 1994, Mubagwa *et al.* 1997), the Na^+_i rise could partially favour Ca^{2+} influx through the reverse mode of the SL-NCE. The results shown in Figure 8 in which the putative selective inhibitor of the reverse mode of SL-NCE KBR (5 μmol L^{-1}) was applied (Satoh *et al.* 2000) are in apparent agreement with such a hypothesis. Nevertheless, in our experimental conditions, SL-NCE could already be inhibited (about 80%) by the presence of 10 mmol L^{-1} BDM (Watanabe *et al.* 2001, 2006). Furthermore, assuming a normal diastolic Ca^{2+}_i of 0.1 μmol L^{-1}, the extreme values achieved for Na^+_i (40 mmol L^{-1}) and membrane potential (−30 mV) reported by other investigators (Bhojani & Chapman 1990, Diederichs 1994, Mubagwa *et al.* 1997) and the Ca^{2+}_o (2.5 μmol L^{-1}) and Na^+_o (135 mmol L^{-1}) levels of the Ca^{2+}-free perfusate in our experiments, the DF calculated for the SL-NCE at the working temperature (25 °C) amounts to +41 mV. The resulting positive DF sign indicates that even under the extreme conditions assumed, Ca^{2+} outward transport prevails and therefore, the diminished increase in H_r found in the presence of KBR (Fig. 8) cannot be attributed to a drug effect on SL-NCE. Towards this end, it has been recently shown

(Santo-Domingo *et al.* 2007) that KBR is also a potent inhibitor of mit-CaU and, therefore, its effect on H_r response to Ca^{2+} withdrawal should be probably associated with it. Thus, as with Ru360, the KBR blockade of mit-CaU depresses mitochondrial Ca^{2+} uptake and Ca^{2+} cycling across the inner mitochondrial membrane and its associated energy usage. Obviously, the addition of CLO to cells in which mitochondrial Ca^{2+} cycling has already been inhibited did not induce any subsequent changes in metabolic rate.

In summary, this work provides information about the energetic consequences of exposing the quiescent adult rat heart to a Ca^{2+}-free medium and evaluates the underlying mechanisms implicated in the observed changes in cardiac basal metabolism. A rise in both Na^+_i and Ca^{2+}_i induces an increased energy expenditure associated with resting state maintenance. In addition to activation of the Na^+ pump, the altered Na^+ and Ca^{2+} homeostasis trigger a mitochondrial Ca^{2+} cycling that it is not detected under normal Ca^{2+}_i diastolic levels. Although far from physiology, the experimental condition here analysed partially mimics findings reported under pathological situations such as heart failure in which enhanced levels of both diastolic Na^+_i and Ca^{2+}_i have also been observed (Baartscheer *et al.* 2003, Bers & Despa 2006, Baartscheer & van Borren 2008). Therefore, under such pathological conditions, the heart must invest part of its chemical energy to fuel processes associated with intracellular Na^+ and Ca^{2+} handling, making more expensive the maintenance of its function.

Conflict of interest

There are no conflicts of interest.

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