Naunyn-Schmied Arch Pharmacol (2011) 383:563–571
DOI 10.1007/s00210-011-0632-z



ORIGINAL ARTICLE

# Mitochondrial $K_{ATP}$ channels participate in the limitation of infarct size by cariporide

**Ignacio Pérez Nuñez · Juliana Fantinelli · Luisa F. González Arbeláez · Susana M. Mosca**

Received: 22 September 2010 / Accepted: 27 March 2011 / Published online: 12 April 2011


**Abstract** The objective of this study is to assess the participation of mitochondrial ATP-sensitive potassium (mito$K_{ATP}$) channels in the cardioprotective effects of the $Na^+/H^+$ exchanger (NHE-1) blocker cariporide in isolated rat hearts. Regional ischemia was induced by occlusion of left anterior descending coronary artery during 40 min followed by 2-h reperfusion (IC). Cariporide (C, 10 μM), or C plus 5-hydroxydecanoate (5-HD, 100 μM, a selective mito$K_{ATP}$ channel inhibitor), or C plus chelerythrine (Chele, 1 μM, a PKC inhibitor), or an opener of mito$K_{ATP}$ channels, diazoxide (Dz, 100 μM) was applied at the onset of reperfusion. Infarct size (IS) and myocardial function were evaluated. The calcium-induced permeability transition pore (mPTP) opening was determined by measuring the light scattering decrease (LSD, a.u.) in isolated mitochondria in the absence and presence of C, C + 5-HD and Dz. IS was 33±2% of the risk area in IC and was significantly diminished by C (15±2%, $p<0.05$), which also improved myocardial function [LVDP=58±5% (IC) vs 80±5% (C)] and blunted LSD [0.80±0.04 (IC) vs 0.51±0.04 (C) a.u.]. 5-HD and Chele were both able to abolish the cardioprotective effects of C on IS. Dz treatment decreased IS and LSD to a similar extent to that produced by C (15±4% and 0.52±0.04 a.u., respectively). The present data suggest that attenuation of mPTP opening after PKC-mediated mito$K_{ATP}$ channel activation is a crucial step for the cardioprotective effects of cariporide.

**Keywords** Cariporide · Mitochondrial $K_{ATP}$ channels · PKC · Infarct size · mPTP

I. P. Nuñez · J. Fantinelli
Fellowship of CONICET, Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 60 y 120, 1900, La Plata, Argentina

L. F. G. Arbeláez
Fellowship of ANPCYT, Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina

S. M. Mosca (✉)
Established Investigator of CONICET, Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina
e-mail: smosca@med.unlp.edu.ar

## Introduction

Calcium overload (Tsujii et al. 2003) and reactive oxygen species (ROS) production (Bolli et al. 1989) contribute to myocardial irreversible injury derived from ischemia and reperfusion. Previous evidence demonstrated that calcium overload is a consequence of reverse $Na^+/Ca^{2+}$ exchanger activation following a $Na^+/H^+$ exchanger (NHE-1)-mediated increase in intracellular sodium (Matsumoto et al. 2003). Consistently, specific NHE-1 blockade before ischemia increased postischemic recovery of myocardial function and decreased infarct size in isolated hearts (Mosca and Cingolani 2000; Hendrikx et al. 1994; Scholz et al. 1995). However, when NHE-1 inhibition was performed during reperfusion, the results were contradictory (Gumina et al. 1998; Klein et al. 2000; An et al. 2001; Hurtado and Pierce 2000; Xiao and Allen 2000). In previous papers, we demonstrated that administration of cariporide only at reperfusion significantly diminished IS (Fantinelli et al. 2006; Garciarena et al. 2011). On the other hand, many studies have shown that during reperfusion an opening of the

mitochondrial permeability transition pore (mPTP) takes place (Halestrap et al. 1998; Crompton 1999; Lesnefsky et al. 2001; Green and Kroemer 2004). In connection with this, a recent study performed by us on isolated mitochondria provided evidence that cariporide attenuates the mPTP opening in a similar manner as cyclosporin A (CsA) (Garciarena et al. 2008). However, recent data by Javadov et al. (2008) showed that the new NHE-1 blocker, AVE, is unable to affect mPTP opening in mitochondrial suspensions. Although other possible targets of NHE-1 inhibitors like the mitochondrial NHE (Hotta et al. 2001; Ruiz-Meana et al. 2003) or the $K_{ATP}$ (mito$K_{ATP}$) channels (Miura et al. 2001) have been suggested, these actions were not conclusively proven. One family of signaling proteins commonly linked to the modulation of ischemia–reperfusion injury is that of protein kinase C (PKC). In this regard, it has been reported that PKCε exerts cardioprotection by inhibiting the mPTP (Baines et al. 2003). Previous papers also showed that activation of PKC potentiates mito$K_{ATP}$ channels opening (Sato et al. 1998; Costa and Garlid 2008).

Thus, the objective of the present study was to determine the possible participation of mito$K_{ATP}$ channels in the protective effects of NHE-1 blockade with cariporide on infarct size, and whether this effect has correlation with an improvement of the mitochondria tolerance to swelling.

## Methods

All procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and to the guidelines laid down by the Animal Welfare Committee of La Plata School of Medicine.

### Isolated heart preparation

Wistar rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight). The heart was rapidly excised and perfused by the non-recirculating Langendorff technique with Ringer's solution containing (in mmol/L): 118 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.35 $CaCl_2$, 20 $NaCO_3H$, and 11.1 dextrose. The buffer was saturated with a mixture of 95% $O_2$–5% $CO_2$, had a pH 7.4, and maintained at 37°C. The conductive tissue in the atrial septum was damaged with a fine needle to achieve atrioventricular block, and the right ventricle was paced at 280±10 beats/min. A latex balloon tied to the end of a polyethylene tube was passed into the left ventricle through the mitral valve; the opposite end of the tube was then connected to a Statham P23XL pressure transducer. The balloon was filled with water to provide an end-diastolic pressure (LVEDP) of 8–12 mmHg, and this volume remained unchanged for the rest of the experiment. Coronary perfusion pressure was monitored at the point of cannulation of the aorta and adjusted to approximately 60–70 mmHg. Coronary flow, controlled with a peristaltic pump, was 11±2 ml/min. Left ventricular pressure (LVP) was acquired by using an analog-to-digital converter and acquisition software (Chart V4.2.3 ADInstruments).

### Experimental protocols

Myocardial infarction was induced after 20-min stabilization by occluding the left anterior descending coronary artery during 40 min followed by 120-min reperfusion. The coronary artery, 3–4 mm from its origin, was encircled by a 6–0 polypropylene suture attached to a small curved needle, and the two ends of the suture were threaded through a length of plastic tubing, forming a snare which could be tightened.

Four experimental protocols were performed (Fig. 1):

1. Ischemic control (IC) ($n$=12): Hearts were reperfused with the preischemic solution.
2. Cariporide (C) ($n$=7): To examine the effects of the inhibition of NHE-1 in reperfusion a specific NHE-1 blocker, cariporide 10 μM was administered during the first 20 min of reperfusion.
3. 5-HD ($n$=6): A specific blocker of mito$K_{ATP}$ channels, 5-hydroxydecanoate (5-HD) 100 μM was administered during the last 5 min of ischemia and during the initial 20 min of reperfusion.

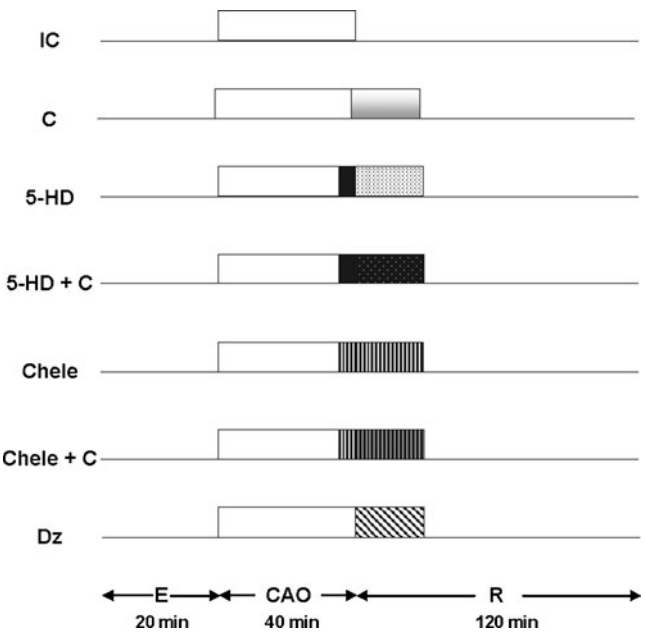


**Fig. 1** Scheme of all the experimental protocols performed in this study. *IC* ischemic control, *C* cariporide, *5-HD* 5-hydroxydecanoate, *Chele* Chelerythrine, *Dz* Diazoxide, *E* estabilization, *CAO* coronary artery occlusion, *R* reperfusion

4. 5-HD + C ($n$=8): To determine the participation of mitoK$_{ATP}$ channels in the effects of C, 5-HD was administered alone during the last 5 min of ischemia and co-administered with C during reperfusion.
5. Chelerythrine ($n$=5): A PKC blocker, chelerythrine (Chele) 1 μM was administered during the last 5 min of ischemia and during the initial 20 min of reperfusion.
4. Chele + C ($n$=6): To determine the participation of PKC in the effects of C, Chele was administered alone during the last 5 min of ischemia and co-administered with C during reperfusion.
5. Dz ($n$=7): An opener of mitoK$_{ATP}$ channels, diazoxide (Dz) 100 μM, was administered during the initial 20-min of reperfusion.

In additional hearts (IC: $n$=9; C: $n$=7; 5-HD: $n$=5; 5-HD + C: $n$=5), lipid peroxidation measured as thiobarbituric acid reactive substance (TBARS) content was analyzed.

## Infarct size determination

Infarct size (IS) was assessed by the widely validated triphenyltetrazoliumchloride (TTC) staining technique (Vivaldi et al. 1985). At the end of reperfusion, the LAD was occluded again, and the myocardium was perfused during 1 min with a 0.1% solution of blue dye. This procedure delineated the nonischemic myocardium as dark blue. The frozen heart was cut into six transverse slices, which were incubated for 5 min at 37°C in a 1% solution of TTC. All atrial and right ventricular tissues were excised. To measure myocardial infarction, the slices were weighed and scanned. The infarcted (pale), viable ischemic/reperfused (red), and nonischemic (blue) areas were measured by computed planimetry (Scion Image 1.62; Scion Corp., Frederick, Maryland, USA). Noninfarcted viable myocardium containing dehydrogenase stained brick red by reacting with TTC, whereas the infarcted tissue remained unstained because of the lack of the enzyme. The area at risk (AAR), the portion of the left ventricle supplied by the previously occluded coronary artery, was identified by the absence of blue dye. Infarct weights were calculated as $(A1\times W1)+(A2\times W2)+(A3\times W3)+(A4\times W4)+(A5\times W5)+(A6\times W6)$, where $A$ is the area of infarct for the slice and $W$ is the weight of the respective section. The weight of the AAR was calculated in a similar fashion. IS was expressed as a percentage of AAR (Suzuki et al. 2002).

## Systolic and diastolic function

Myocardial contractility was assessed by the left ventricular developed pressure (LVDP), obtained on subtracting LVEDP values to the LVP peak values and maximal rise velocity of left ventricular pressure (+dP/dt$_{max}$) values. Data were expressed as percentage of their respective preischemic values. The diastolic function was evaluated by the isovolumic LVEDP.

## Assessment of TBARS

At the end of reperfusion period, hearts were homogenized and centrifuged, and TBARS in the supernatant was determined by a spectroscopic technique (Buege and Aust 1978). The absorbance at 535 nm was measured, and TBARS was expressed in nmol/g tissue weight using an extinction coefficient of $1.56\times10^5$ $M^{-1}cm^{-1}$.

## Isolation of rat heart mitochondria

Hearts were immediately removed from rats, and mitochondria from left ventricle (LV) were isolated as described by Mela and Seitz (1979). Briefly, LV were washed and homogenized in ice-cold isolation solution (IS) consisting of 75 mM sucrose, 225 mM mannitol, and 0.01 mM EGTA neutralized with Trizma buffer at pH 7.4. After the tissue pieces were settled, the entire supernatant was discarded and fresh IS (5 ml) was added, and the mixture was transferred to a hand homogenizer. Proteinase (0.8 mg, bacterial, type XXIV, Sigma, formerly called Nagarse) was added just before starting the homogenization procedure. The whole homogenization procedure took no longer than 14 min in two steps of 7 min each (with 5 ml addition of fresh IS each). The homogenate was carefully transferred after each step to a polycarbonate centrifuge tube. After 5 min of $480\times g$ of centrifugation to discard unbroken tissue and debris, the supernatant was centrifuged at $7{,}700\times g$ for 10 min to sediment the mitochondria. The mitochondrial pellet was washed twice with IS and the last one with suspension solution (IS without EGTA) at $7{,}700\times g$ for 5 min each. Mitochondria protein content was evaluated by Bradford method (1976) using bovine serum albumin as standard. An average of 10 mg mitochondrial protein/ml was obtained from one rat heart.

## $Ca^{2+}$-induced mPTP opening

The ability of mitochondria to resist swelling was assessed by incubating isolated mitochondria in a buffer containing (in mmol/L): 120 KCl, 20 MOPS, 10 Tris HCl, and 5 $KH_2PO_4$ (Baines et al. 2003), adjusted to pH=7.4. After 5-min preincubation, the mitochondria energized with the addition of 6 mM succinate were induced to swell with 200 μM $CaCl_2$. If the mPTP is open in the presence of $Ca^{2+}$ loading, solutes will be free to enter the inner matrix, causing the mitochondria to swell. These changes are observed as decreases of light scattering and followed

using a temperature-controlled Hitachi F4500 spectrofluorometer operating with continuous stirring at excitation and emission wavelengths of 520 nm (Facundo et al. 2007). Light scattering decrease (LSD) was calculated for each sample by taking the difference of scattered light between before and after the addition of $CaCl_2$, in the presence and absence of the pharmacological agents tested (C, 5-HD, 5-HD + C and Dz). Each drug was incubated during 5 min before $Ca^{2+}$ addition. In order to relate mPTP opening to decreased light scattering, we added cyclosporine (CsA) 1 μM to inhibit mPTP or to abolish any observed reduction. To assess the possible action of C on mitochondrial NHE, the changes of LSD were measured in isolated mitochondria incubated at pH=6.03. The LSD recovery after 5 min of $Ca^{2+}$ addition was also analyzed.

### Statistical analysis

Data are given as means ± SE. The statistical analysis was performed using repeated measures of one-way analysis of variance (ANOVA) with the Newman–Keul's test for multiple comparisons among groups. Values of $p<0.05$ were considered to be significant.

## Results

Forty minutes of regional ischemia followed by 2-h reperfusion in rat hearts caused an IS of ~30% of the RA (Fig. 2). The RA for all interventions was similar and represented ~32% of the left ventricle. A significant reduction in IS was obtained when 10 μM C was added to the perfusate during the first 20 min of reperfusion (Fig. 2), a result in accordance with previous reports by our and other laboratories (Fantinelli et al. 2006; Garciarena et al. 2011; An et al. 2001; Hurtado and Pierce 2000; Xiao and Allen 2000). To determine the participation of mitoK$_{ATP}$ channels and PKC in the cardioprotective action of C, we next explored whether 5-HD or Chele could affect IS when co-administered with C. As shown in Fig. 2, combination of 5-HD + C and Chele + C abolished the effects of C on IS, suggesting that both mitoK$_{ATP}$ channels and PKC are involved in the protective effect of C against reperfusion injury. Interestingly, administration of the mitoK$_{ATP}$ channels opener Dz during reperfusion mimicked the protective effect of C on IS reinforcing the notion that activation of these channels may be underlying the beneficial effect of C.

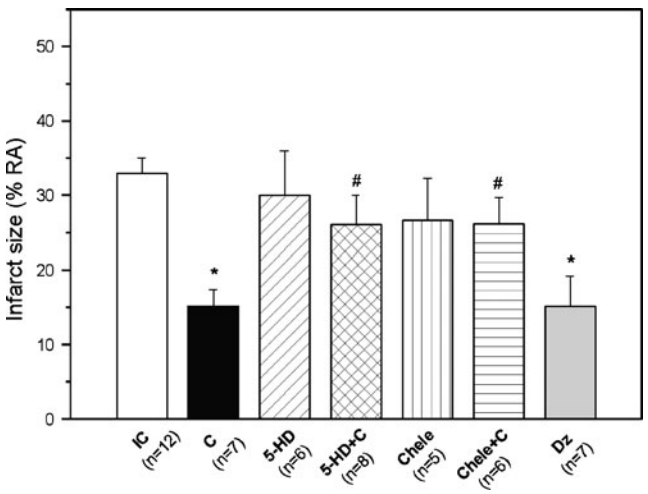


**Fig. 2** Infarct size, measured at the end of reperfusion period and expressed as percentage of risk area (RA), in ischemic control (IC) and in hearts treated with cariporide (C) 10 μM, 5-hydroxydecanoate (5-HD) 100 μM, chelerythrine (Chele) 1 μM, the combinations 5-HD + C and Chele+C and diazoxide (Dz) 100 μM. Note that the hearts treated with C and Dz showed a significant lower infarct size than IC hearts. The effect of C was abolished by mitoK$_{ATP}$ channels blockade and PKC inhibition. *$p<0.05$ with respect to IC; #$p<0.05$ with respect to C

Figure 3 shows the effects of all interventions on systolic myocardial function. At the end of the reperfusion period, LVDP decreased to ~58% of the preischemic value in ischemic control hearts. Postischemic recovery was significantly improved by C reaching a value of ~80% of the preischemic control. This improvement was abolished either by mitoK$_{ATP}$ channels blockade or PKC inhibition. Treatment with 5-HD alone did not modify the postischemic recovery of ischemic control hearts. Although the postischemic recovery after treatment with Chele appears to be lower than that observed in untreated hearts, the values of LVDP and + dPdt$_{max}$ were not statistically different. Similar to C, the mitoK$_{ATP}$ channels opener Dz improved myocardial recovery of systolic function during reperfusion (Fig. 3, upper panel). A similar pattern was observed when + dP/dt$_{max}$ values were analyzed (Fig. 3, lower panel). On the other hand, diastolic stiffness was also evaluated by measuring LVEDP that was initially settled at ~10 mmHg at the end of the stabilization period in the different experimental groups. LVEDP significantly increased in ischemic control hearts reaching a value of ~26 mmHg after 2 h of reperfusion. This increase was canceled by C treatment, a protective effect that disappeared when C was co-incubated with 5-HD or Chele (Fig. 4).

Given that ROS generation and the consequent tissue damage that they promote may be responsible for myocardial reperfusion injury (Zweier 1988; Ambrosio et al. 1991), we next determined the impact of the pharmacological interventions on myocardial TBARS concentration, used as an index of lipid peroxidation. TBARS concentration was 7.63± 0.48 nmol/g in ischemic control hearts, a value that was significantly reduced by 10 μM C (2.99±0.32 nmol/g).

Similar to the results observed when evaluating IS or LV function, co-administration of C with 5-HD reduced the protective effect of the NHE-1 inhibitor reaching a value of 5.25±0.72 nmol/g ($n=5$, $p<0.05$). These results revealed

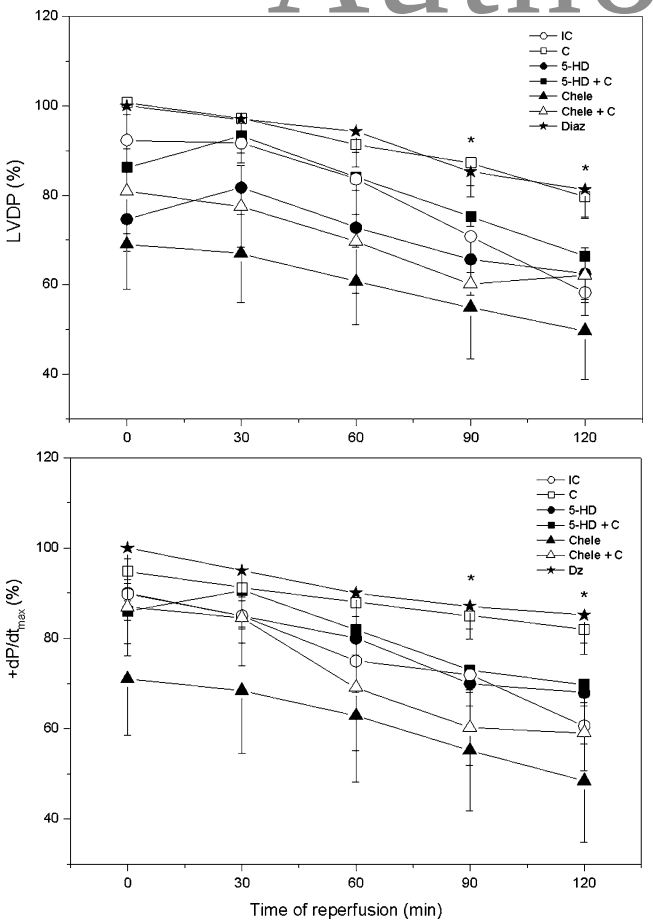


**Fig. 3** Changes of left ventricular developed pressure (LVDP) and maximal velocity of contraction ($+dP/dt_{max}$) during the reperfusion period, expressed as percentage of preischemic values in ischemic control (IC) and in hearts treated with cariporide (C) 10 μM, 5-hydroxydecanoate (5-HD) 100 μM, chelerythrine (Chele) 1 μM, the combinations 5-HD + C and Chele + C and diazoxide (Dz) 100 μM. Observe that C significantly improved the postischemic recovery of myocardial systolic function at the end of reperfusion period, and this beneficial effect was annulled when $mitoK_{ATP}$ channels were blocked by 5-HD and when PKC was inhibited by Chele. *$p<0.05$ with respect to IC

that the final effects of C, IS reduction, and improvement of postischemic myocardial function were associated to a diminution of oxidative damage, and this reinforces the idea of a direct or indirect oxidative effect of C, which we (Fantinelli et al. 2006; Garciarena et al. 2008) and others (Javadov et al. 2008) previously reported.

Figure 5a shows a typical light scattering trace over time produced by the addition of 200 μM $Ca^{2+}$ to a mitochondrial suspension obtained from control rats in the absence and presence of C, 5-HD, 5-HD + C, and Dz. Figure 5b shows values of light scattering decrease (LSD) from all interventions. C limited mitochondrial swelling to ~60% of untreated mitochondria. The presence of 5-HD did not modify the $Ca^{2+}$ induced mitochondrial swelling but annulled the effect of C. Once again, Dz mimicked the effect of C. These results would suggest that C and Dz partially prevent mPTP opening through a common pathway that involves $mitoK_{ATP}$ channels opening. As shown in the figure, the decreases of LS observed after $Ca^{2+}$ addition in absence and presence of drugs did not change over time suggesting that $Ca^{2+}$ efflux is somehow inhibited. It is important to highlight that none of the drugs modified light scattering under basal conditions (results not shown).

Considering that mNHE activity increases at acidic pH, the LSD after $Ca^{2+}$ addition were examined in mitochondria suspended at pH=6.03. As shown in Fig. 6, the addition of C did not modify the mitochondrial swelling obtained with $Ca^{2+}$ addition, and though LSD partially recovered, it did not reach baseline values. The similar LSD after $Ca^{2+}$ obtained in mitochondria suspended at pH 7.4 and 6.03 is suggesting that the acidic conditions out of the organelle do not modify the $Ca^{2+}$-mediated mPTP opening. However, after 5 min of $Ca^{2+}$ addition the LS of control mitochondria returned to baseline values while that of mitochondria treated with C stabilized at a value of ~60%. These data suggest that C attenuates $Ca^{2+}$ efflux and favors the mPTP opening.

## Discussion

The results of this study suggest that the selective NHE-1 inhibitor C limits IS and improves postischemic recovery of

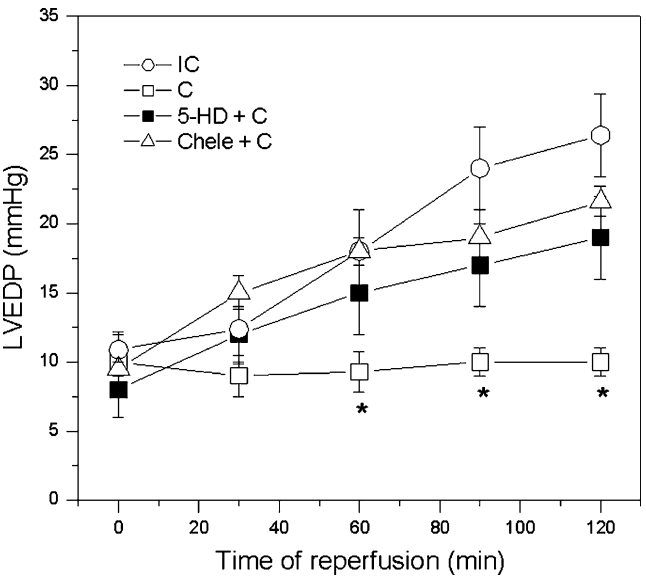


**Fig. 4** Changes of left ventricular end diastolic pressure (LVEDP) during the reperfusion period, expressed in mmHg, in ischemic control (IC) and in hearts treated with cariporide (C) 10 μM, and the combinations 5-HD (100 μM) + C and Chele (1 μM) + C. Observe that C significantly attenuated the increase of diastolic stiffness detected in IC hearts. This beneficial effect was annulled when $mitoK_{ATP}$ channels were blocked by 5-HD and when PKC was inhibited by Chele. *$p<0.05$ with respect to IC

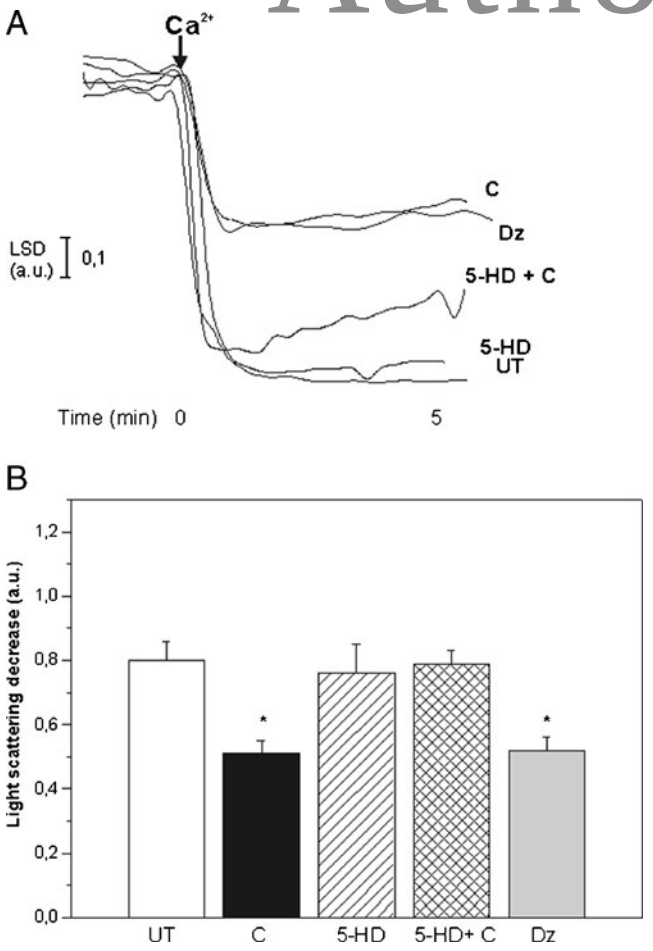


**Fig. 5** **A** Typical traces produced by 200 μM $Ca^{2+}$ addition to mitochondrial suspensions untreated (UT, $n$=10) or treated with cariporide 10 μM (C, $n$=8), 5- hydroxydecanoate 100 μM (5-HD, $n$=6), 5-HD + C ($n$=6) and diazoxide 100 μM (Dz, $n$=7) incubated at pH=7.40. **B** Mean values of the light scattering decreases (LSD), measured as indicated in "Methods" section and expressed in arbitrary units (a.u.) after all the interventions. Note that C, similar to Dz, decreased the LSD $Ca^{2+}$-mediated and it was abolished when mito$K_{ATP}$ channels were blocked by 5-HD. *$p$<0.05 with respect to UT

contractility by attenuation of mPTP opening through a mechanism that involves PKC-mediated mito$K_{ATP}$ channels activation.

The cardioprotective property of C has been shown in various experimental models of ischemia–reperfusion injury (Mosca and Cingolani 2000; Hendrikx et al. 1994; Scholz et al. 1995; Gumina et al. 1998; Klein et al. 2000). In this regard, we recently demonstrated that C treatment applied only at reperfusion is able to diminish IS and improve postischemic cardiac recovery by decreasing ROS formation (Fantinelli et al. 2006; Garciarena et al. 2011), but the exact mechanism by which this compound blunted ROS formation was not described at that time. It is important to remember that the NHE-1 plays an important role in maintaining intracellular pH being at the same time one of the most important routes of $Na^+$ entry into the cell (Lazdunski et al. 1985), therefore indirectly controlling cytosolic $Ca^{2+}$ homeostasis. During ischemia, the development of intracellular acidosis activates the exchanger and with the progress of ischemia the cell becomes overloaded with $Na^+$, which favors reverse $Na^+/Ca^{2+}$ exchange, thus increasing intracellular $Ca^{2+}$ content. Early during reperfusion the NHE is further activated leading to $Ca^{2+}$ overload which is a pathognomonic sign of irreversible ischemia/reperfusion injury (Shen and Jennings 1972; Allen et al. 1993).

Recent data from Karmazyn's group show that the beneficial actions of NHE-1 inhibitors are mediated by a diminution of mitochondrial $Ca^{2+}$ overload subsequent to the sarcolemmal NHE-1 blockade and not by a direct mitochondrial action (Javadov et al. 2008). The mNHE has

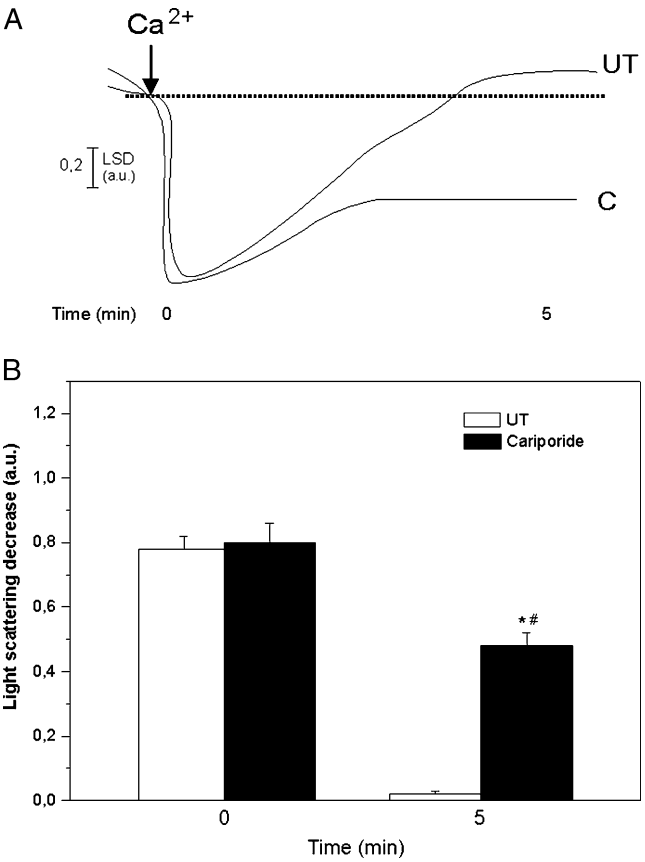


**Fig. 6** **A** Typical traces produced by 200 μM $Ca^{2+}$ addition to mitochondrial suspensions untreated (UT, $n$=6) or treated with cariporide 10 μM (C, $n$=6) incubated at pH=6.03. **B** Mean values of the light scattering decreases (LSD) at time 0 and 5 min after $Ca^{2+}$ addition, expressed in arbitrary units (a.u.). Observe that C did not modify the LSD detected in untreated mitochondria at time 0. After 5 min of $Ca^{2+}$ addition mitochondria treated with C still showed LSD in comparison to control mitochondria which returned to the initial light scattering. This pattern indicates that the inhibition of mNHE decreases the $Ca^{2+}$ efflux and maintains the mitochondrial swelling actions associated to a deleterious instead of a beneficial effect of C. *$p$<0.05 with respect to UT; #$p$<0.05 with respect to time 0

been considered as a possible target of NHE-1 inhibitors but this action remains controversial. In this regard, Kapus and coworkers (1988) reported a lack or weak inhibitory action of amiloride analogs and derivatives on mNHE. Under physiological conditions, the mNHE introduces cytosolic $H^+$ into the mitochondrial matrix in exchange for mitochondrial $Na^+$. Therefore, a decrease in the exchanger activity should reduce $H^+$ and increase $Na^+$ concentration in the mitochondrial matrix. This increase in $Na^+$ would decrease the inwardly directed $Na^+$ gradient, affecting the mitochondrial $Na^+/Ca^{2+}$ exchanger, other factors being constant (Murphy and Steenbergen 2008). A decrease in $Ca^{2+}$ efflux from the mitochondria would increase mitochondrial $Ca^{2+}$ concentration and favor rather than reduce mPTP opening. These events could be taking place in our preparation of isolated mitochondria incubated at acidic pH. In these conditions, the greater activity of mNHE and consequent $Na^+/Ca^{2+}$ exchanger could lead to increased $Ca^{2+}$ efflux, avoiding the mitochondrial $Ca^{2+}$ overload. This would allow the mitochondria to restore its volume, as evidenced by the return of light scattering to their baseline. Thus, the mPTP opening would be only transitory. However, in the presence of C the light scattering maintains a value of approximately 60% of baseline suggesting that the blockade of mNHE is attenuating $Ca^{2+}$ efflux and favoring the mPTP opening. Then, these effects could not explain the cardioprotective action of C.

Other mitochondrial actions of C like on mito$K_{ATP}$ channels have already been reported but the information is controversial (Hale and Kloner 2000; Miura et al. 2001; Xiao and Allen 2003). To test the possibility that C might exert a positive effect on mito$K_{ATP}$ channels, 5-HD, a blocker of these channels was given before C. This treatment inhibited the IS-reducing capability of C. These data are in accordance with those reported by Miura et al. (2001) in which the limitation of IS and the improvement of recovery of contractility produced by either C or ethyl isopropylamiloride were prevented by 5-HD. Opposite results were obtained by Xiao and Allen (2003) and Hale and Kloner (2000) the first one demonstrating additive cardioprotective effects of NHE blockade and activation of mito$K_{ATP}$ channels and the other showing that the reduction of IS obtained after C was not modified by 5-HD treatment. The most important difference between these studies and the present resides on the administration time of the drugs. In the majority of the papers mentioned C was given before ischemia whereas in that, in our case, it was administered at the beginning of reperfusion, mimicking the ischemic postconditioning cardioprotective intervention (Zhao et al. 2003) and offering multiple advantageous for clinical therapy of postinfarction.

Our data reveal that mito$K_{ATP}$ channels are involved in the protection afforded by C. The fact that the opener of mito$K_{ATP}$ channels Dz, administered at the beginning of reperfusion, produces a similar limitation of IS than C reinforces the idea that these channels are possible mitochondrial targets of the NHE-1 inhibitor. This hypothesis is also supported by the experiments performed in isolated mitochondria which show that both C and Dz similarly reduce the calcium mediated mPTP opening-, effect canceled in the presence of 5-HD. Then, these data suggest that C could be exerting a direct or indirect effect on mito$K_{ATP}$ channels favoring its opening.

ROS are known to be produced in large quantities in the first few minutes of post-ischemic reperfusion (Mason et al. 2000). The oxidative injury due to increased ROS formation leads to changes in membrane permeability, membrane lipid bilayer disruption and functional modification of various cellular proteins. In fact, oxidative stress is associated with modifications of membrane phospholipids and proteins leading to peroxidation and oxidation of thiol groups (Molavi and Mehta 2004). Previous experiments performed in our laboratory (Fantinelli et al. 2006) show that C and a ROS scavenger, mercaptopropionylglycine (MPG) similarly reduce the IS and suggest that ROS-mediated NHE-1 activation is the responsible mechanism for the reperfusion injury. In that paper, we also showed that both drugs (C and MPG) diminished the lipid peroxidation in a similar proportion, suggesting that C possesses an "antioxidant action". In the present study, an attenuation of lipid peroxidation in hearts treated with C was observed, effect that was partially abolished by 5-HD

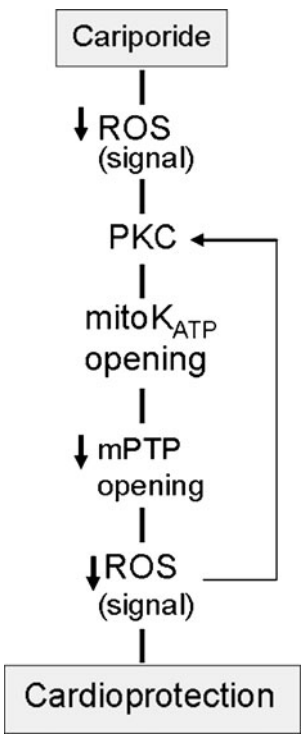


**Fig. 7** Scheme of proposed mechanism for cardioprotection by cariporide

indicating that mitoK$_{ATP}$ channels are involved in the diminution of oxidative damage achieved by that NHE-1 inhibitor. Pain et al. (2000) showed that opening of mitoK$_{ATP}$ channels acted not as a mediator of protection but rather as a signal transduction event in the trigger phase by causing the mitochondria to produce ROS, which then acted as second messengers for redox signaling. Thus, ROS play two roles at the same time, one inducing reperfusion injury and the other carrying a protective signal as it was previously demonstrated in the postconditioning phenomenon (Penna et al. 2006). ROS have been suggested to induce mitoK$_{ATP}$ channels opening (Das and Sarkar 2003). On the other hand, Costa and Garlid (2008) demonstrated that mitoK$_{ATP}$ channels opening produces ROS, then ROS activate PKC and this kinase promotes phosphorylation-dependent mitoK$_{ATP}$ channels opening. Our results show that PKC inhibition annulled the reduction of IS afforded by C and indicate that PKC is involved in the signaling pathway leading to cardioprotection. Although it was previously demonstrated that $Ca^{2+}$ can activate PKC (Wang and Ashraf 1999), our data assign a more important role for ROS as responsible for that activation. Then, we could propose that the order of events triggered by C is first ROS reduction maintaining an enough level for the protective role, followed by PKC activation by ROS and opening of mitoK$_{ATP}$ channels, and finally ending with inhibition of mPTP formation and reduction of ROS release, which may activate PKC and initiate a positive feedback loop that may be responsible for the lasting effect after C is no longer present. A scheme of the proposed mechanism for cardioprotection by C is shown in Fig. 7.

The results of the present study suggest that C administered at the beginning of reperfusion decreases the myocardial IS and the mPTP opening by PKC and mitoK$_{ATP}$ channels-dependent mechanism. We could speculate that intermittent mitoK$_{ATP}$ channels opening through PKC activation by a low ROS concentration—insufficient to produce oxidative damage—acts as a trigger for cardioprotection afforded by C.

**Acknowledgments** This work was supported in part by the grant PICT 1046 from Agencia Nacional de Promoción Científica y Técnica of Argentina to Dr Susana M Mosca.

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