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CaMKII inhibition protects against necrosis and apoptosis in irreversible ischemia–reperfusion injury

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Abstract

Objectives: Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has been implicated in the regulation of cardiac excitation–contraction coupling (ECC) as well as in apoptotic signaling and adverse remodeling. The goal of the present study is to investigate the role of CaMKII in irreversible ischemia and reperfusion (I/R) injury.
Methods: Isovolumic Langendorff perfused rat hearts were subjected to global no-flow I/R (45 min/120 min), and isolated myocytes were subjected to a protocol of simulated I/R (45 min simulated ischemia/60 min reoxygenation) either in the absence or presence of CaMKII inhibition [KN-93 (KN) or the CaMKII inhibitory peptide (AIP)].
Results: In I/R hearts, an increase in CaMKII activity at the beginning of reperfusion was confirmed by the significantly increased phosphorylation of the Thr^{17} site of phospholamban. In the presence of KN, contractile recovery at the end of reperfusion was almost double that of I/R hearts. This recovery was associated with a significant decrease in the extent of infarction, lactate dehydrogenase release (necrosis), TUNEL-positive cells, caspase-3 activity, and an increase in the Bcl-2/Bax ratio (apoptosis). In isolated myocytes, both KN and AIP prevented simulated I/R-induced spontaneous contractile activity and cell mortality. Similar results were obtained when inhibiting the reverse mode Na^+/Ca^{2+} exchanger (NCX) with KB-R7943, sarcoplasmic reticulum (SR) function with ryanodine and thapsigargin, or SR Ca^{2+} release with tetracaine. In contrast, overexpression of CaMKII decreased cell viability from 52±3% to 26±2%.
Conclusions: Taken together, the present findings are the first to establish CaMKII as a fundamental component of a cascade of events integrating the NCX, the SR, and mitochondria that promote cellular apoptosis and necrosis in irreversible I/R injury.

Keywords: CaMKII; Ischemia/reperfusion injury; Apoptosis; Necrosis; Myocardium

1. Introduction

Coronary ischemic disease is one of the most important causes of adult mortality in Western countries. This reflects the lack of effective therapies targeted to the molecular basis of ischemia/reperfusion (I/R) damage and is the main trigger for the enormous effort focused on elucidating the basic biological processes within the ischemic myocardium. In the past few years evidence has grown indicating that modification of reperfusion conditions is critical for reducing the damage to the ischemic heart. It was shown that the early phase of reperfusion is of particular relevance because cardiac injury (*reperfusion injury*) may occur in this phase, in addition to the injury produced by the preceding ischemia [1]. Reperfusion injury appears, however, to be a complex phenomenon, and its

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underlying mechanisms are still unclear [2–5]. Recent experiments from our laboratory described for the first time a cascade of events that involves enhanced Ca^{2+} influx, CaMKII activation and phosphorylation of phospholamban (PLN) at the Thr^{17} residue that is beneficial for the recovery of contractility and intracellular Ca^{2+}(Ca_i^{2+}) handling in reversible I/R injury, also known as stunned heart [6–8]. The protective effects of the phosphorylation of PLN residues in the stunned heart are also supported by experiments in which the phosphorylation of Ser^{16} of PLN by PKG increased the contractile recovery of isolated myocytes submitted to simulated I/R [9].

CaMKIIδ is the predominant CaMKII isoform in the heart, and its splice variant δ_C resides in the cytosol. In addition to its well documented role in the regulation of cardiac excitation–contraction-coupling [10–12], CaMKIIδ_C has been implicated in apoptotic signaling [13–16] and in mediating the transition to heart failure [17]. These latter reports, together with recent evidence indicating that both necrosis and apoptosis cause cardiac injury during I/R, could suggest a detrimental role of CaMKII-dependent phosphorylations that may contribute to the irreversible I/R injury. These results are in clear contrast to the beneficial effect previously described for reversible I/R injury [6–8]. The present experiments were undertaken to explore whether CaMKII activity is either protective or detrimental in irreversible I/R disease, and in the last case, whether CaMKII increases cardiac injury by favoring necrosis and/or promoting apoptosis.

2. Methods

2.1. Animals

Wistar male rats (200–300 g) were anaesthetised by intra-abdominal injection of sodium pentobarbitone (35 mg (kg body weight)$^{-1}$). Animals used in this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85–23, revised 1996).

2.2. Langendorff perfusion

Isolated hearts were perfused according to the Langendorff technique at constant temperature (37 °C), coronary flow (12–14 ml/min) and heart rate (240 beats/min), as previously described [6,7]. The mechanical activity of the heart was assessed by passing into the left ventricle (LV) a latex balloon connected to a pressure transducer (ADInstruments MLT9580, CO) and filled with aqueous solution to achieve a left ventricular end-diastolic pressure of approximately 10 mm Hg. LV contractility was evaluated by the developed pressure (LVDP) and the maximal rate of pressure development ($+dP/dt$).

2.3. Experimental protocol

After stabilization, hearts were perfused for 10 min and then, normothermic global ischemia was produced by interruption of the coronary flow for a period of 45 min. Coronary perfusion was then restored for 120 min. CaMKII inhibition was achieved by treating the hearts, 10 min before ischemia and during the first 10 min of reperfusion, with 2.5 μM of the CaMKII inhibitor KN-93 (KN). This concentration of KN produced a slight albeit significant decrease in basal contractility (12.9±3.9%, $n=12$). At indicated times during the I/R protocol, hearts were either freeze-clamped and stored at −80 °C for biochemical assays or submitted to the procedures described below.

2.4. Infarct size

After the reperfusion period, hearts were perfused with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 7 min and immersed in this solution for another 7 min. The hearts were frozen at −20 °C for 1 h and then cut into six transverse slices (2 mm thick) along the long axis of the LV, from apex to base. Infarct areas were enhanced by storage in 10% formaldehyde solution for 48 h before measurement. The infarct area was calculated by computer planimetry (Scion Image, Scion Corp, USA) and expressed as a percentage of the total area of the heart [18].

2.5. LDH determination

Cardiac injury was assessed by measuring the level of the LDH released in the perfusion effluent of the first 10 min of reperfusion. LDH was assessed by an enzymatic method (Roche, Mannheim).

2.6. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end (TUNEL) labeling

Myocardial slices obtained from the middle of the long axis of the LV were fixed in buffered formalin and processed for histological examination. TUNEL assay was performed in deparaffinized sections (5 μm thick) with an ApopTag kit (Chemicon International) according with the supplier's instructions. Stained sections counterstained with hematoxylin were captured with a digital RGB video camera (Evolution VF, Qimaging, Canada) attached to a microscope (Olympus BX-50, Tokyo). Twenty areas at high magnification (objective 40×) were randomly chosen in each stained section and approximately 500 myocytes were analyzed per section. Images were processed by a digital image analyzer program (Image-Pro Plus V6.O, Media Cybernetics Silver Spring, MA) and percentage of TUNEL-positive myocytes was calculated by two blinded independent investigators. Cardiac myocytes were identified following morphologic (nuclei with elliptical shape and striated cytoplasm) and morphometric parameters [19].

2.7. Myocyte isolation

Myocytes were isolated by enzymatic digestion and kept in a HEPES buffered solution at room temperature until used, as previously described [20]. Unless otherwise specified, experiments were performed at 37 °C.

2.8. Simulated I/R

Myocytes were plated in HEPES buffered solution on 35 mm cell culture dishes. Simulated ischemia was performed according to Louch et al. [21], with minor modifications. Ischemia (45 min) was reproduced by a buffer exchange to ischemia-mimetic solution (in mM: 123 NaCl, 20 HEPES, 0.9 NaH_2PO_4, 8 KCl, 0.5 $MgSO_4$, 2.5 $CaCl_2$ and 20 Na-lactate, gassed with 100% N_2 and pH adjusted to 6.2 with NaOH). A 100% N_2 gas phase was layered over the experimental chamber during simulated ischemia. "Reperfusion" was initiated by returning to the initial HEPES buffered solution and removal of the N_2 gas phase. Assessment of cell viability and apoptosis was performed after 60 min of reperfusion.

2.9. Assessment of cell viability and apoptosis

Cell viability was assessed by trypan blue exclusion assay. At the end of the simulated I/R protocol, 20 μl of 0.4% trypan blue was added to the culture dish. After ~5 min of equilibration, cells were counted under microscope. Cells were also classified as viable or nonviable according to their length-to-width ratio (>3 were considered viable [22]). Apoptosis was measured using the caspase-3 fluorescent substrate, Phiphilux (OncoImmunin; Gaithersburg, MD). 2.5 μM Phiphilux was added to each culture dish at the start of reoxygenation and allowed to incubate for 1 h at 37 °C. Myocytes were gently washed once and imaged under a fluorescence microscope.

2.10. Recombinant adenoviruses

Two first-generation type 5 recombinant adenovirus, were used. Ad.βgal carries the β-galactosidase and the green fluorescent protein (GFP) genes and Ad.CaMKII, carries both the CaMKIIδ_C and the GFP genes, each under separate cytomegalovirus promoters.

2.11. Culture and adenovirus infection of adult ventricular cardiomyocytes

After isolation, cells were resuspended in DMEM medium containing (g/l): 0.0176 ascorbic acid, 2 BSA, 0.4 L-carnitine, 0.66 creatine, 0.62 taurine, 50 U/ml penicillin and 50 U/ml streptomycin, and counted. Myocytes plated at a density of 5×10^4 cells/ml were infected with adenoviruses at a multiplicity of infection of 100 and cultured for 48 h. After this period, cells were used to verify transgene expression and virus transfection efficiency and for protocols of simulated I/R.

2.12. Transgene expression and virus transfection efficiency

Transgene expression was monitored by GFP fluorescence at an excitation wavelength of 480 nm. Virus transfection efficiency was verified by Western blotting.

2.13. Indo-1 fluorescence and cell shortening measurements

Isolated myocytes were loaded with Indo-1/AM (17 μM for 9 min) and Indo-1 fluorescence and cell shortening was determined as previously described [19,23]. Ratio of Indo-1 emission (410 and 490 nm) was taken as an index of

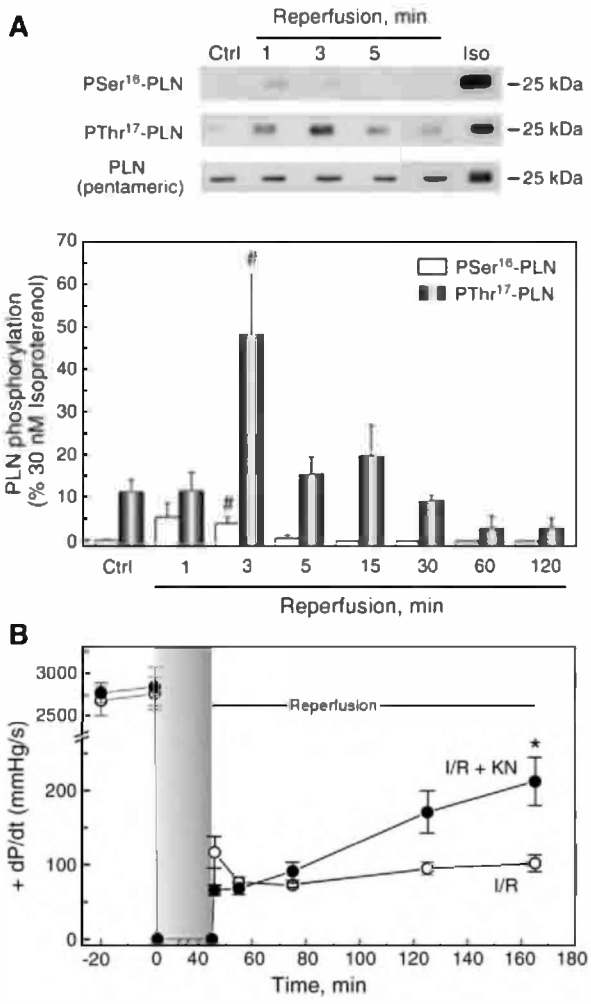

Fig. 1. Increased phosphorylation of Thr^{17} of PLN and mechanical recovery during reperfusion. A. Representative immunoblots showing the phosphorylation of PLN sites ($PSer^{16}$-PLN and $PThr^{17}$-PLN) of hearts submitted to different times of reperfusion after the 45 min of ischemia and of non-ischemic hearts perfused in the absence (Ctrl) and the presence of 30 nM isoproterenol (Iso), run in parallel and used as an internal positive control. A blot probed against PLN demonstrates equal protein loading at all the different times showed. Overall results of 3–11 hearts show that the phosphorylation of Thr^{17} of PLN increased at the onset of reperfusion, indicating an enhancement of CaMKII activity. $n=3$ only correspond to time points greater that 60 min, whereas all other time points consist of at least 6 experiments. PLN phosphorylation was expressed as percentage of Ser^{16} and Thr^{17} phosphorylation induced by Iso. #$p<0.05$ *vs.* Ctrl. B. Time course of maximal rate of rise of pressure development ($+dP/dt$) of hearts submitted to the I/R protocol, in the presence and absence of CaMKII inhibition (2.5 μM KN). Note the expanded ordinate scale between 0 and 250 mm Hg to better visualize the effect of KN during reperfusion. Results are average of 4 hearts in each group. *$p<0.05$ *vs.* I/R in the absence of KN.

the Ca_i^{2+}. This group of experiments was run at room temperature.

2.14. Sample preparation for Western blot

Homogenates, cytosolic fractions and SR membrane vesicles were prepared from the pulverized ventricular tissue of the perfused hearts [6,24,25] and whole-cell lysates were obtained from isolated myocytes [20]. Protein was measured by the method of Bradford using bovine serum albumin as standard.

2.15. Electrophoresis and Western blot analysis

Proteins from cardiac homogenates (80 μg, caspase-3), cytosolic fractions (30 μg, Bcl-2, Bax), SR membrane vesicles (15 μg, PLN) and cell lysates (80 μg, CaMKII, actin) were separated on appropriate Laemmli SDS-polyacrylamide gels (buffer: 25 mM Tris–HCl, 190 mM Glycine and 0.1% SDS) and transferred to PVDF membranes as previously described [6,24]. Blots were probed with antibodies raised against Ser^{16} or Thr^{17}-phosphorylated PLN (Badrilla, Leeds), PLN (ABR), active-caspase-3, phospho-CaMKII (Chemicon International), Bcl-2, Bax (Santa Cruz Biotechnology) and actin (Sigma). Immunoreactivity was visualized by peroxidase-based chemiluminescence detection kit (Amersham Biosciences). The signal intensity of the bands in the immunoblots was quantified by densitometry using Image J software (NIH, USA).

2.16. Statistical analysis

Data are expressed as means±SEM. Unpaired or paired Student *t* test or ANOVA were used for statistical comparisons

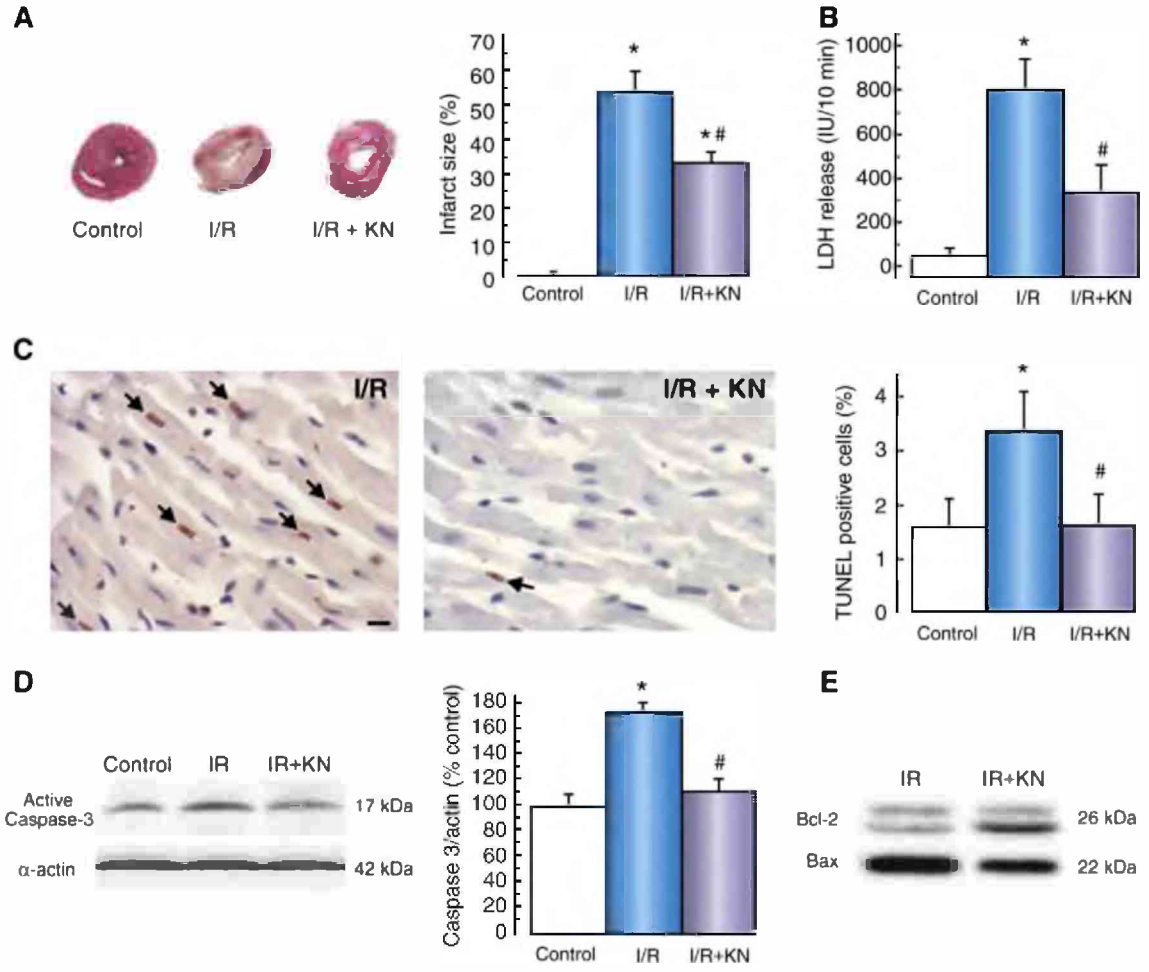

Fig. 2. CaMKII inhibition diminished I/R-induced infarct size, necrosis and apoptosis. A. Cross-sections of TTC staining in non-ischemic hearts (control) and hearts submitted to irreversible I/R protocol in the absence (I/R) or presence of 2.5 μM KN (I/R+KN). The bar graph on the right depicts the overall results of 4 independent experiments in each group. B. Lactate dehydrogenase (LDH) release during the first 10 min of reperfusion of control ($n=9$), I/R ($n=10$) and I/R+KN ($n=5$) hearts. C. TUNEL staining in LV transverse sections obtained at the end of the I/R protocol in the absence or presence of KN. CaMKII inhibition reduced the number of TUNEL positive cells (brown nuclei with arrows). The calibration bar represents 10 μm. The bar graph shows pooled data from $n=3$–7 experiments. D. Representative blots of active-caspase-3 and α-actin in control, I/R and I/R+KN hearts. The bar graph shows the overall data of $n=4$–5 experiments. E. Representative blots of Bcl-2/Bax protein expression determined in hearts subjected to I/R in the absence and presence of 2.5 μM KN. Similar results were obtained in 3 additional experiments. Data are expressed as mean±SEM, *$p<0.05$ *vs.* control; #$p<0.05$ *vs.* I/R.

when appropriate. Differences were considered significant at $p<0.05$.

3. Results

3.1. Time course of the phosphorylation of PLN and the mechanical recovery during the irreversible I/R injury

Fig. 1A shows the phosphorylation of PLN residues at different times during reperfusion after 45 min of global ischemia in perfused rat hearts. Phosphorylation of Ser^{16} of PLN was slightly increased at the beginning of reperfusion reaching significant levels at 3 min. Thr^{17} phosphorylation was also increased at 3 min of reperfusion and then slowly decayed towards levels lower than controls. The increase in the phosphorylation of Thr^{17} site of PLN indicates an enhanced activity of CaMKII, triggered by the onset of reperfusion. Previous reports have demonstrated in rat and mouse hearts that this reperfusion-induced Thr^{17} phosphorylation could be diminished by the CaMKII inhibitor, KN-93 [6,8]. Fig. 1B shows that contractile recovery during the reperfusion period was extremely depressed, in agreement with what has been already reported after long ischemic periods of global ischemia [26]. This modest recovery was

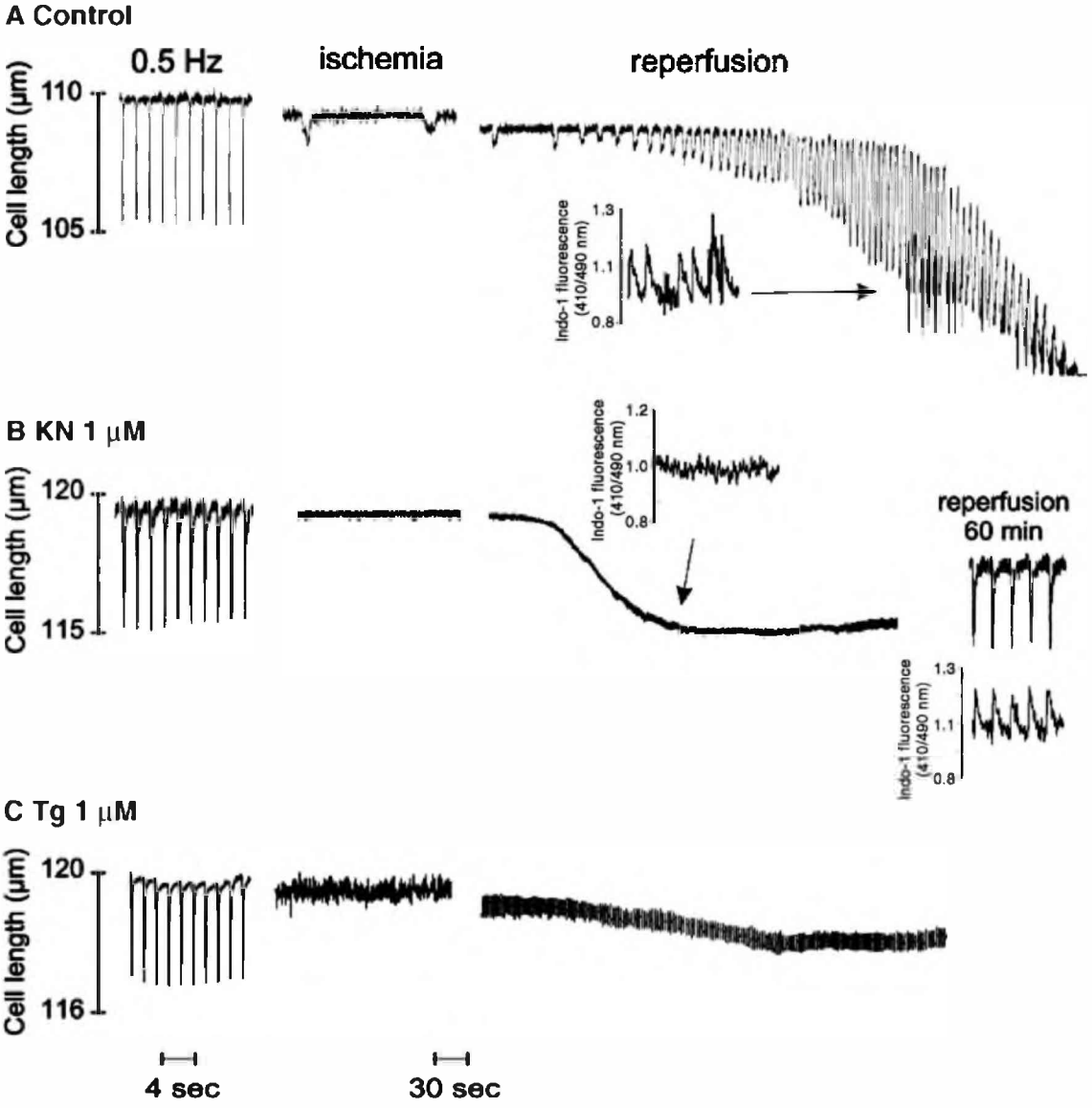

Fig. 3. CaMKII inhibition prevents spontaneous oscillations in isolated myocytes. Simultaneous recordings of cell shortening and Ca_i^{2+} in adult rat cadiomyocytes during a protocol of I/R. The tracings on the left show steady state contraction at 0.5 Hz in a control cell (A), and in cells treated either with KN (B) or Tg (C). During the ischemic period (middle tracings), unpaced control cells show sporadic spontaneous contractions which are absent in the KN and Tg treated cells. At the start of reperfusion (right tracings) control cells begin to beat profusely and asynchronously in association with large Ca_i^{2-} oscillations (inset). These spontaneous events are absent in KN and Tg treated cells. Returning electrical stimulation to 0.5 Hz after 60 min of reperfusion in KN-treated cells elicits typical contractile activity and Ca^{2+} transients (panel B, far right).

almost doubled however, in the presence of CaMKII inhibition. Absolute values of $+dP/dt$ previous to ischemia were 3191.88±215.52 mm Hg/s in non-treated *vs.* 2839.97± 220.35 mm Hg/s in KN-treated hearts.

3.2. CaMKII inhibition decreases myocardial infarct size and LDH release

Myocardial infarct size was determined after the I/R protocol in the presence and the absence of CaMKII inhibition (2.5 μM KN) in perfused hearts. Fig. 2A shows representative TTC stained myocardial slices and overall results of control, I/R and I/R+KN treated hearts. The inhibition of CaMKII greatly reduced the infarct size produced by I/R. The level of LDH release was also assessed to evaluate necrosis. Total LDH release was reduced by approximately 57% in hearts submitted to I/R in the presence of 2.5 μM KN, when compared with non-treated hearts (Fig. 2B). These findings indicate reduced necrosis in rat hearts perfused with the CaMKII inhibitor. Taken together the above results point to a deleterious effect of CaMKII in the irreversible I/R injury.

3.3. CaMKII inhibition attenuates I/R-induced apoptosis

To study whether CaMKII inhibition influences the degree of apoptosis evoked by I/R, TUNEL staining was performed in control and I/R hearts, in the absence and presence of KN. KN reduced I/R-induced apoptosis as indicated by the reduction in the number of TUNEL positive nuclei (Fig. 2C). Since activation of caspases is a critical step in apoptosis, caspase-3 activity was also determined in KN-treated and non-treated hearts subjected to the I/R protocol. Fig. 2D shows that KN significantly reduced caspase-3 activity induced by I/R. We also assessed the effect of KN on Bcl-2 and Bax protein expression (Fig. 2E). The presence of KN during I/R significantly increased the Bcl-2/Bax ratio with respect to I/R hearts (3.13±0.51 *vs.* 1.73±0.19, $n=10$).

3.4. I/R causes intracellular Ca^{2+} oscillations in isolated myocytes

In isolated myocytes subjected to simulated I/R, we measured cell shortening and Ca_i^{2+} (Fig. 3). Prior to the I/R protocol, myocytes responded to electrical stimulation (0.5 Hz) with the expected normal steady state contractions and showed only sporadic spontaneous contractions during the ischemic period, when stimulation was stopped. In 5 out of 6 myocytes studied, reperfusion evoked multiple and large spontaneous contractions and Ca_i^{2+} oscillations, that culminated in membrane rupture and cell death. These multiple spontaneous events were prevented by 1 μM KN (Fig. 3B). Furthermore, KN-treated cells presented contractile activity and Ca_i^{2+} cycling when electrical stimulation was resumed at the end of the reperfusion period, indicating that cell viability was preserved. Spontaneous contractions were also absent during reperfusion in thapsigargin (Tg, Fig. 3C) or tetracaine (not shown) treated cells, providing evidence that these events are driven by release of Ca^{2+} from an overloaded SR.

3.5. CaMKII-inhibition prevents cell death in isolated cardiac myocytes submitted to simulated I/R

The protective effect of CaMKII inhibition was tested in cultured myocytes submitted to a protocol of simulated I/R. Fig. 4 shows that after 160 min of incubation under normoxic conditions, approximately 80% of the cells show rod-shape morphology. Simulated I/R reduced the number of viable myocytes to 50% and treatment of the myocytes with 1 μM KN

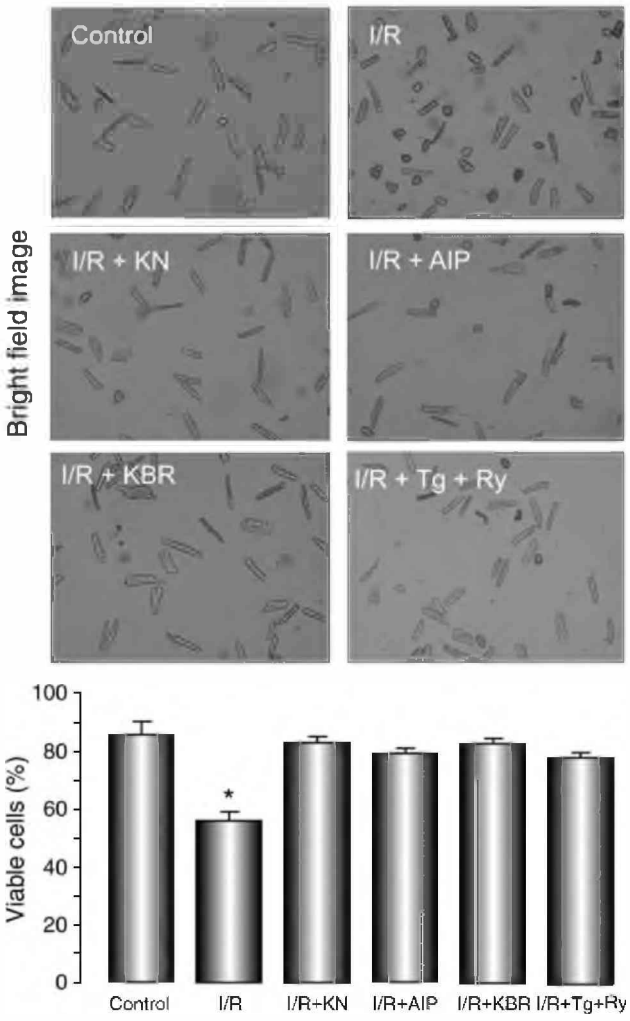

Fig. 4. Inhibition of CaMKII, reverse NCX mode and SR, prevent cell death induced by simulated I/R. Transmitted images of control myocytes and myocytes submitted to a protocol of simulated I/R, either in the absence (I/R) or the presence of inhibitors of CaMKII, 1 μM KN-93 (IR+KN) or 1 μM AIP (I/R+AIP), of the inhibitor of the reverse mode NCX, 5 μM KBR (I/R+KBR) or inhibitors of SR function 1 μM Tg+1 μM Ry (I/R+Tg+Ry). The representative images clearly show that the simulated I/R protocol produced a vast number of nonrod shaped/nonviable cells which were largely absent when simulated I/R was induced after CaMKII inhibition, reverse mode NCX blockade or inhibiting SR function. The overall results of these experiments are shown in the bar graph bellow. Data are mean±SEM from 5 independent experiments using cells form 5 hearts.

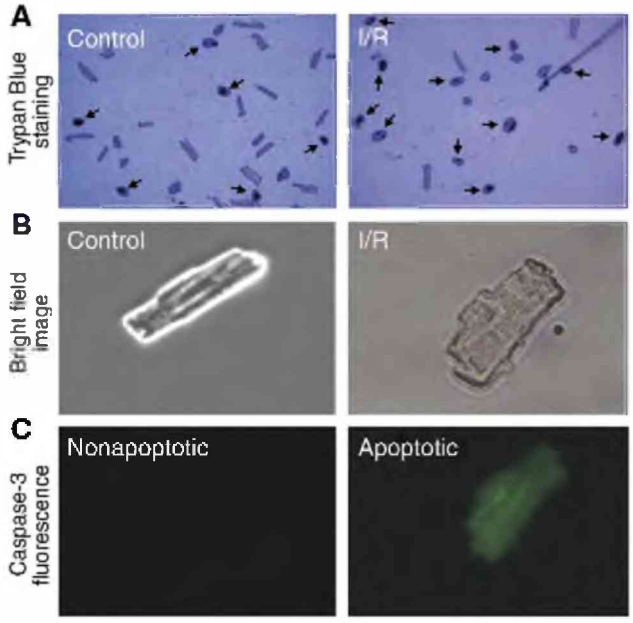

Fig. 5. Simulated I/R induces both necrosis and apoptosis in isolated myocytes. A: Representative bright field images showing that simulated ischemia induces necrosis, as evidenced by the increased number of trypan blue positive cells (arrows) B: Bright field images of representative myocytes under control, normoxic conditions (left) or after 45 min of ischemia and 120 min of reperfusion (right) and C: corresponding fluorescent images of the same myocytes as in B, demonstrating apoptotic caspase-3 activation.

prior and throughout the I/R protocol, prevented cell mortality. Similar results were obtained in the presence of 1 μM of the more specific CaMKII inhibitory peptide, AIP. The results on this figure further show that I/R-induced cell death could also be prevented by inhibiting Ca^{2+} influx via the NCX using 5 μM KB-R7943 (KBR), a specific inhibitor of the reverse NCX mode, or by inhibiting SR function using 1 μM ryanodine (Ry) plus 1 μM thapsigargin (Tg). Furthermore, I/R-induced cell mortality was also prevented by blocking ryanodine receptors with 1 mM tetracaine. Taken together, these results indicate that both the NCX and the SR are key components of the signaling pathway leading to cell death. The doses of the inhibitors used were selected based on their IC_{50} for their specific target proteins. These inhibitors did not affect cellular viability when applied alone under control conditions and during the entire duration of the I/R protocol. In control experiments, we found that KBR, KN and AIP, at the concentrations used, did not significantly affect basal contractility and Ca^{2+} transient kinetics, whereas Ry+Tg produced a significant decrease in basal contractility and amplitude of the Ca^{2+} transient and a slowing of Ca^{2+} transient decay. Tetracaine decreased contractility to barely perceptible levels.

3.6. Simulated I/R causes necrosis and apoptosis in isolated cardiac myocytes

In a separate group of experiments, we evaluated whether apoptosis was involved in myocyte death induced by simulated I/R. Fig. 5A shows that after the simulated I/R protocol, cell viability was largely reduced. Most cells died from necrosis, as it was clearly evidenced by the increase in trypan blue positive myocytes. However, as shown in panels B and C, apoptotic signaling was also induced by I/R in isolated

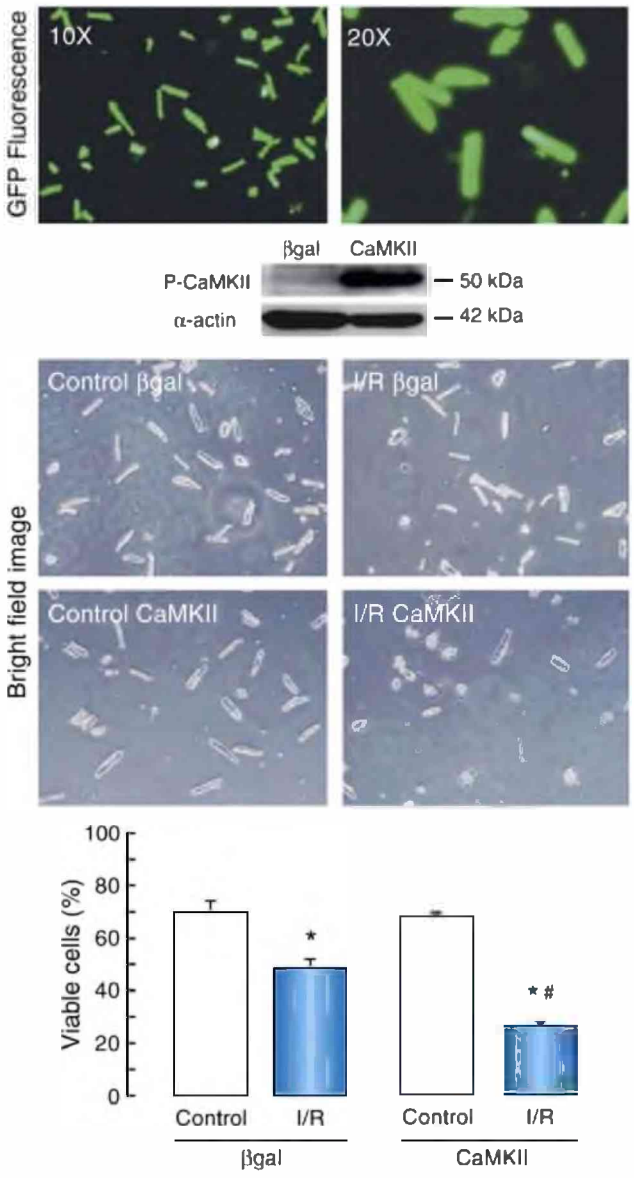

Fig. 6. Overexpression of CaMKII enhanced cell death in isolated myocytes submitted to simulated I/R. Fluorescent images of myocytes, 48 h after infection with Ad.CaMKII. 100% of the myocytes showed GFP fluorescence indicating the co-expression of CaMKII. The representative blots below, of phospho-CaMKII and α-actin confirm the overexpression of Ad.CaMKII *vs.* Ad.βgal infected cells. The transmitted images show that similar to the observation in fresh cells, I/R reduced cell viability in βgal overexpressing cells to approximately 50% (I/R βgal). In CaMKII overexpressing cells, the negative impact of I/R on cell viability was greatly enhanced as evidenced by the increased number of dead cells (I/R CaMKII). The bar graph below depicts the overall results of cell viability assessed at the end of the reperfusion period. Three different fields with an average of 30 to 60 cells were counted from triplicate dishes for each experimental condition. Data are mean±SEM from 4 independent experiments from 4 hearts. #$p<0.05$ *vs.* I/R βgal. *$p<0.05$ *vs.* control.

myocytes as indicated by the increase in caspase-3 activity detected using a fluorescent caspase-3 substrate (5±1% control *vs.* 21±3% I/R). These results indicated that the decreased viability of isolated myocytes after the simulated I/R protocol, was due at least in part, to apoptotic cell death.

3.7. Overexpression of CaMKII enhances cardiac cell death in isolated myocytes submitted to simulated I/R

The participation of CaMKII in I/R-induced cell death was confirmed in experiments of isolated myocytes overexpressing CaMKII (Fig. 6). After 48 h of incubation, 100% of rod-shaped myocytes, infected with either Ad.βgal or Ad. CaMKII virus, presented a robust expression of the reporter gene GFP, visualized by fluorescence microscopy and of CaMKII protein expression as confirmed by Western blotting. Similar to the results obtained in fresh cells, simulated I/R reduced cell viability by 50% in myocytes overexpressing βgal. However, CaMKII overexpression rendered cells more susceptible to I/R injury, as indicated by the significantly larger number of dead cells. The bar graph bellow depicts the overall results of these experiments.

4. Discussion

Our results indicate that activation of CaMKII at the beginning of reperfusion has a deleterious effect in the irreversible I/R injury, participating in a cascade of events that lead to apoptosis and necrosis. To our knowledge, this is the first report demonstrating the protective effect of CaMKII inhibition against both necrosis and apoptosis, caused by irreversible I/R injury. This effect was independent of the preparation and experimental protocol used, since it was observed in intact hearts submitted to global I/R and in isolated myocytes submitted to simulated I/R.

4.1. Role of the NCX

Previous studies from our and other laboratories indicate that the increase of Ca^{2+} influx which occurs at the beginning of reperfusion is mainly mediated by the reverse mode of the NCX [1,4,6,7]. The involvement of the NCX, both in forward and reverse modes, in the production of SR Ca^{2+} overload and apoptosis has been also previously reported [27,28]. The present results confirm these findings, since the deleterious effect of reperfusion was abolished in isolated myocytes by blocking the reverse NCX mode with the specific inhibitor KBR. Moreover, we previously showed that CaMKII-dependent phosphorylation of PLN at the onset of reperfusion, could be prevented by treating the perfused hearts with KBR. These findings indicate that Ca^{2+} influx via the NCX can induce CaMKII activation and PLN phosphorylation [6]. The present results further suggest that Ca^{2+} influx via the reverse NCX mode could be the major source of Ca^{2+} for the activation of CaMKII in the setting of irreversible I/R.

4.2. Role of CaMKII

In the I/R hearts, CaMKII inhibition reduced infarct size in association with a significant recovery of contractility during reperfusion, with respect to the untreated hearts. Moreover, CaMKII inhibition reduced LDH release, caspase-3 activity and the number of TUNEL positive cells, and increased Bcl-2/Bax ratio, indicating that CaMKII inhibition prevents both necrosis and apoptosis. The protective role of CaMKII inhibition against necrosis was confirmed in isolated myocytes, where we observed a decrease in cell mortality after I/R, when cells were treated with either KN or the more specific CaMKII inhibitor, AIP. The deleterious role played by CaMKII in I/R was further supported by the evidence that CaMKII overexpression in isolated myocytes exacerbated cellular death. The fact that the protection introduced by CaMKII inhibition was obtained in isolated myocytes, using two different inhibitors that did not affect basal contractility or Ca^{2+} dynamics, would rule out the possibility of unspecific effects of the drugs used. Previous studies have shown CaMKII to be pro-apoptotic under conditions of enhanced β1-adrenergic stimulation, or enhanced Ca^{2+} influx through L-type Ca^{2+} channels [15,16]. Recent results by Yang et al. [14], using isolated ventricular myocytes from transgenic mice overexpressing a highly specific CaMKII inhibitory peptide, have further confirmed the apoptotic effects of this kinase *in vivo* during excessive catecholamine stimulation and its possible contribution to left ventricular adverse remodeling. In this study, we extend the concept of the deleterious role of CaMKII to another pathophysiological situation, the irreversible I/R injury. Furthermore, the results shown herein are the first to describe a role for CaMKII in necrotic cell death induced by I/R in addition to its pro-apoptotic effect.

4.3. Role of the SR and mitochondria

Although our results do not reveal which is the target of CaMKII action, they do suggest a possible signalling cascade in which the SR and the mitochondria appear to be involved. The important role played by the SR and the release of Ca^{2+} through RyR2 in the modulation of apoptosis and necrosis during reperfusion is supported in the present work by two types of experimental evidence: 1) The protocol of simulated I/R in isolated myocytes produced large spontaneous contractions and Ca^{2+} oscillations upon reperfusion, typical of SR Ca^{2+} overload. These Ca^{2+} waves were absent after inhibition of SR Ca^{2+} uptake (Tg), SR Ca^{2+} release (tetracaine) or CaMKII (KN). These results indicates that the spontaneous contractions and Ca^{2+} oscillations are driven by the release of Ca^{2+} from an overloaded SR and furthermore, that they are dependent on CaMKII activity; 2) Blockade of SR Ca^{2+} uptake and release with Ry+Tg, reduced myocyte death. Collectively, our findings directly relate the increase in SR Ca^{2+} load and release with the production of CaMKII-dependent cell death. The involvement

of the SR in the apoptotic and necrotic pathways and the increased SR Ca^{2+}-load as a fundamental step in triggering both death processes has been previously reported [4,29]. Indeed, experimental evidence linked the apoptotic effect of CaMKII, observed under conditions of enhanced Ca^{2+} influx through L-type Ca^{2+} channels, to an increased SERCA2a activity. These experiments suggested an apoptotic pathway in which the phosphorylation of Thr^{17} site of PLN constitutes a key mechanism for SR Ca^{2+} overload, which would then lead to SR Ca^{2+} leak and mitochondrial Ca^{2+} uptake [16]. Although in our studies the phosphorylation of Thr^{17} was examined and used as "a marker" of CaMKII activation at the onset of reperfusion, the experimental evidence indicates that this phosphorylation might represent an important step in the series of events leading to SR Ca^{2+} overload. Indeed, both phenomena, phosphorylation of Thr^{17} and Ca^{2+} overload (6 and the present results), could be prevented by CaMKII inhibition. Our results also showed that CaMKII inhibition can prevent the decrease in the Bcl-2/Bax ratio and the significant increase in caspase-3 activity produced by I/R. Bax and/or Bak can translocate to mitochondria in the presence of apoptotic stimuli, like an increase in cytosolic Ca^{2+}, and they oligomerize to form pores, allowing cytochrome c release and caspase-3 activation. This action is prevented by the antiapoptotic members of the Bcl-2 family, like Bcl-2 and Bcl-xL [30]. Our results would then indicate the involvement of mitochondria in the signalling pathway leading to apoptosis, in addition to the role of the SR [30].

Taken together, the present findings suggest a cascade of events involving the reverse mode NCX, the SR and the mitochondria, where the activation of CaMKII plays a critical role in the signalling to apoptosis and necrosis. In this scenario, it is tempting to speculate that both apoptosis and necrosis induced by I/R share the same trigger *i.e.* SR Ca^{2+} overload and release, induced by CaMKII activation. Indeed, previous studies have shown that necrosis and apoptosis induced by I/R can share a common signalling pathway [31].

Overall, the present study shows that CaMKII inhibition can effectively protect against I/R-induced necrosis and apoptosis, highlighting the relevance of CaMKII inhibition as an important therapeutic tool, to be applied during reperfusion to reduce myocyte death by I/R injury. In addition, the present results, in conjunction with previous findings from our laboratory [6–8], indicate a dual role of CaMKII in the I/R. We have previously shown that CaMKII mediates a cardioprotective effect after a short period of ischemia (stunned heart), allowing an improved contractile response through the phosphorylation of the Thr^{17} site of PLN. In contrast, the present results demonstrate that CaMKII activation is detrimental in the irreversible I/R injury, inducing necrosis and apoptosis of cardiac myocytes. The reason for this dual effect is unknown although a similar dual action for CaMKII has been reported in other pathological conditions. Indeed, the activation of CaMKII during acute ß1-adrenergic stimulation has beneficial effects on heart function, since it mediates the positive inotropic and relaxant actions of these agents [24], while CaMKII activity becomes an apoptotic signal in sustained β1-adrenergic stimulation, [15]. Although speculative, our results suggest the possibility that the previous myocardial ischemic history of the heart determines the protective or detrimental action of this kinase.

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