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Direct, pleiotropic protective effect of cyclosporin A against simulated ischemia-induced injury in isolated cardiomyocytes

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Abstract

Cyclosporin A is an immunosuppressor that prolongs graft survival but its use is limited by cardiotoxicity. The effects of cyclosporin A on several functional and biological characteristics were thus evaluated in rat cardiomyocytes in normal conditions and in a substrate-free, hypoxia–reoxygenation model of ischemia–reperfusion. Cyclosporin A (100 and 1000 ng/ml) did not induce cardiocytotoxicity in basal conditions. Simulated ischemia gradually decreased and then blocked the spontaneous electromechanical activity. Cyclosporin A at 100 and 1000 ng/ml permitted the maintenance of electromechanical functions that were abolished in control cells. Cyclosporin A also improved the post-"ischemic" functional recovery. Cyclosporin A reduced the "ischemia"-induced lactate dehydrogenase and troponine I releases and the successive rises in heat shock protein mRNA observed after "ischemia" and reoxygenation. Moreover, cyclosporin A improved the resumption of the mitochondrial function. To conclude, cyclosporin A displayed a direct, pleiotropic protection of isolated cardiomyocytes against physiological, metabolic, structural and stress signaling changes induced by ischemia–reperfusion mimicked in vitro.

Keywords: Neonatal rat cardiomyocyte; Cyclosporin A; Ischemia–reperfusion; Electrophysiology; Cell contraction; Protein release; Heat shock protein; Mitochondria

1. Introduction

Heart transplantation is a surgical therapy for irreversible end-stage heart failure caused by ischemic, valvular, or congenital cardiomyopathies. Allograft success depends on the efficiency of the immunosuppressive treatment (Rovira et al., 2000). The introduction of cyclosporin A has increased survival after cardiac transplantation. Cyclosporin A exerts its action by inhibiting T cell activation. Briefly, cyclosporin A binds to cyclophilin and then to calcineurin, a serine/threonine phosphatase regulated by calcium/calmodulin (Matsuda and Koyasu, 2000). By inhibiting the phosphatasic activity of calcineurin, cyclosporin A affects the translocation of various factors including activator protein-1 (AP-1), nuclear factor of activated T cells (NF-AT) and nuclear factor-κB (NF-κB), which are involved in the transcription of cytokine genes (interleukin 2 and 4) (Matsuda and Koyasu, 2000).

However, the use of cyclosporin A is limited by a variety of side effects, such as nephrotoxicity and hypertension. Although cardiotoxic effects are also mentioned (Owunwanne et al., 1993), few reports addressed cyclosporin A functional effects on the myocardium. Nevertheless, data suggest that cyclosporin A is able to alter heart mechanical properties and the myocardial sensitivity to calcium (Banijamali et al., 1993; Kingina et al., 1991). Some authors described mitochon-

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drial and enzymatic damages in the myocardium in the presence of cyclosporin A (Hutcheson et al., 1995; Millane et al., 1994). In pressure overload, cyclosporin A could display a complex effect on compensatory myocardial hypertrophy with an increased risk of decompensation (Meguro et al., 1999). Cyclosporin A may also favor the development of fibrosis in the graft (Karch and Billingham, 1985). However, the notion of a direct myocardial action of cyclosporin A has been recently challenged, since these secondary undesirable effects have been attributed to the vehicle of cyclosporin A (Tatou et al., 1996; Sanchez et al., 2000), although this issue is still debated (Jurado et al., 1998).

In contrast, several studies showed that cyclosporin A could protect the myocardium against ischemia, when applied before or at the beginning of ischemia (Borutaite et al., 2003; Sharkey et al., 2000; Schneider et al., 2003; Weinbrenner et al., 1998). This beneficial influence of cyclosporin A is attributed to its capability to alleviate isolated rat heart injury by preserving mitochondria (Crompton, 1999; Suleiman et al., 2001), although mitochondrial effects of cyclosporin A have been often investigated in respect of reperfusion damage only (DiLisa et al., 2001). Conversely, cyclosporin A has been reported to augment the ischemia–reperfusion injury to endothelial cells (Azizian et al., 2004).

Only few studies address the effects of cyclosporin A on isolated cardiomyocytes. In cardiac cell preparations, cyclosporin A could act on transmembrane movements of Ca^{2+} (Park et al., 1999). Cyclosporin A could directly modify the contractility, the morphology and the cytoskeletal structure in chick embryo cardiomyocytes (Kolcz et al., 1999). Moreover, cyclosporin A has no effect on collagen metabolism of isolated myocardial fibroblasts in culture (Eleftheriades et al., 1993), suggesting that cyclosporin A may have no direct effect on the interstitial fibrosis induction suggested in vivo. Otherwise, direct evidences of an influence of cyclosporin A on the ischemic cardiac muscle cells themselves are scarce, although an effect of the drug on mitochondrial function of anoxic-reoxygenated cardiomyocytes has been also suggested (Ganote and Armstrong, 2003; Griffiths et al., 2000).

Therefore, the aim of the work was to explore the specific effects of cyclosporin A on the physiological functions and on various metabolic and biochemical characteristics of cultured newborn rat cardiomyocytes in basal conditions and in a substrate-free hypoxia–reoxygenation model of ischemia–reperfusion. The data obtained indicated that cyclosporin A did not display by itself adverse effects on isolated cardiomyocytes. Additionally, the present work shows for the first time that this immunosuppressive agent exerted direct, pleiotropic protective effects against metabolic, structural and stress signaling changes induced by an in vitro simulated ischemia–reperfusion.

2. Materials and methods

2.1. Cell culture preparation

This investigation conformed to the authorization 00775 from the French government, which agrees with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The cardiac muscle cell cultures were prepared using the method previously described by Grynberg et al. (1986). Briefly, ventricles from 2- to 4-day-old Wistar rat (Janvier, le Genest St Isle, France) were removed, washed and dissociated using seven dissociation cycles of 10 min each with trypsin at 0.1% (Difco, Detroit, MI, USA). The cells were collected by centrifugation and were suspended in standard culture medium composed of Ham's F-10 medium (Cambrex, Verviers, Belgium) supplemented with 10% fetal calf serum and 10% human serum. The cells were then seeded in 60×15 Petri dishes (Falcon Primaria 3802, Becton Dickinson, Oxnard, CA, USA) and incubated during 30 min and then during 2 h in a humidified incubator (5% CO_2, 19% O_2 and 76% N_2) to allow early adherence of fibroblasts. Cells were diluted at a final density of 2×10^6 cell/dish and incubated at 37 °C. The cultures were changed 24 h after seeding and then every 2 days.

2.2. Cell function measurements

The transmembrane potentials were obtained using conventional microfiber microelectrodes filled with 3.0 M KCl and connected to a unity gain amplifier (Biologic VF 180, Grenoble, France). The contractions were photometrically recorded by the use of a video cell motion detector, the optical signal corresponding to the cell shortening being either upward or downward, depending upon the light intensity of the cell area focused at the relaxed state (Athias and Grynberg, 1987). A universal counter (Harvard Apparatus, South Natick, MA, USA) measured the spontaneous rate of either action potentials or contractions. The electromechanical signals were displayed on the screen of a digital storage oscilloscope (Gould DSO 1604, Ilford, Essex, UK). These signals were stored on magnetic tapes using a digital data recorder (Biologic DRA-400) and transcribed on paper chart with an oscillographic recorder (Graphtec WR3320, Yokohama, Japan).

The following action potential and contraction parameters were measured: maximal diastolic potential, action potential amplitude, action potential duration (at 80% repolarization from top), action potential rate, contraction duration at 80% full contraction, shortening time measured from 20% to 80% full contraction amplitude.

2.3. Ischemia–reperfusion simulation

The ischemia–reperfusion insult was simulated in vitro through a substrate-free hypoxia–reoxygenation model,

which has been previously described and validated (Chevalier et al., 1990; Fantini et al., 1990; Laubriet et al., 2001; Tissier et al., 2002). One hour before the experiments, the culture was placed in a glucose-free, Puck's F saline solution, which was covered with a layer of paraffin oil. All experiments were done in static bath conditions. For electrophysiological studies, the Petri dish was placed in a water-heated microchamber (36±0.1 °C), which was secured on the stage of an inverted, phase-contrast microscope (Diavert, Leica, Wetzlar, Germany). The microchamber was covered with an optically clear enclosure that allowed the control of atmosphere surrounding the cell culture during the course of the experiments, with facilities for continuous electrophysiological recordings and periodic drug addition and bath sampling. During control normoxia and reoxygenation, the cell culture was continuously flushed with air. Hypoxia was obtained by a flow of nitrogen. The PO_2 values in the bath during normoxia, hypoxia and reoxygenation periods were 101.4±2.1, 20±6.3 and 89.3±9.3 mm Hg, respectively. For biochemical studies, we used a 24-well, benchtop temperature-controlled manifold similar to the 1-well chamber used for the electrophysiological studies (Chevalier et al., 1990).

2.4. Cell viability assays

To evaluate the extent of cardiomyocyte injury, we analyzed the release of lactate dehydrogenase (LDH) and of troponine I in the extracellular bath (Li et al., 2004). The LDH activity was measured using a commercial spectrophotometric assay (Cytotox96, Promega, Madison, WI, USA). The LDH release was expressed as a percentage of the total LDH activity (released LDH and intracellular LDH). Troponin I concentration was determined in 150 μl samples of external bath by a fluorometric enzyme immunoassay analyzer (Stratus CS, Dade Behring, Newark, DE).

The mitochondrial function was investigated by the use of the (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) test (Sigma, St. Louis, MO, USA), which reflects the metabolic status of the cells (Gomez et al., 1997). MTT was reduced by mitochondrial reductases that led to the formation of a blue formazan. The quantification was done by spectrophotometry at 570 and 630 nm. MTT (500 μl, 1 mg/ml) was added in each well and placed 1 h at 37 °C. Adding 1 ml of isopropanol and 1 N HCl stopped the reaction, and the dishes were then replaced at 37 °C until complete solubilization.

2.5. RT-PCR

The steady state level of the inducible heat shock protein 70 (HSP70i) gene transcripts has been evaluated by a reverse transcriptase polymerase chain reaction (RT-PCR) assay using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence as internal standard (Laubriet et al., 2001). After treatment, we lysed cells in 800 μl of guanidium isothiocanate and phenol solution 5 (Tri reagent). The first-strand cDNA was synthesized by random priming from one microgram of total RNA using the MMLV reverse transcriptase (Invitrogen, Karlsruhe, Germany). The following PCR primers were used: 5′-ACCACAGTCCATGCGATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for GAPDH, 5′-TGCTGACCAAGATGAAG-3′ and 5′-AGAGTCGATCTCCAGGC-3′ for HSP70i. The conditions for amplification were as follows: denaturation at 95 °C for 45 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1.5 min. The PCR products were electrophoresed in a 2% ethidium bromide stained agarose gel for quantification of fluorescence by image analysis under UV light (Molecular Analyst Software, Biorad).

2.6. Pharmacology

Cyclosporin A (Calbiochem, San Diego, USA) was diluted at 1:40 or 1:400 in methanol. These stock solutions were prepared and kept at −20 °C until use. Aliquots of stock solutions were added to the experimental bath (5 ml) with a microsyringe (Hamilton, Bonaduz, Switzerland) to obtain final concentrations of 100 and 1000 ng/ml (0.08 and 0.8 μM, respectively). The homogeneous distribution of cyclosporin A in the bath was achieved by gentle mixing. Previous control experiments have shown that the methanol alone had no effects at these dilutions (data not shown).

2.7. Protocols

The experimental protocols performed were the following: (i) long-term chronic exposure: cyclosporin A was added in the standard culture medium during 24 h and 48 h and then the electrical and contractile activities were recorded during 1 h in the same medium; (ii) simulated ischemia: electrical and contractile activities were recorded during control normoxia (1 h), during 1 h after cyclosporin A addition in normoxia, followed by 2.5 h simulated ischemia and 1.5 h reoxygenation. These protocols were repeated for MTT tests with cyclosporin A at the concentration of 1000 ng/ml.

2.8. Statistical analysis

To study the effects of cyclosporin A at 100 and 1000 ng/ml on electromechanical parameters, with or without simulated ischemia, 4 culture dishes obtained from different culture preparations were used in each protocol. In each of these dishes, the recordings and the related measurements and calculations were carried out from 3 different areas of the cultured cell monolayer. The resulting individual data were submitted to a 2-way analysis of variance, in which dishes were considered as random factor and treatment as experimental factors. For cardiomyocyte viability and metabolic data, each experimental protocol was performed

on separate sets of 4 dishes. The data obtained were compared to control groups of 4 dishes. Differences were considered as significant at $P<0.05$.

3. Results

3.1. Basic characteristics of rat cardiomyocytes

Cardiomyocytes cultured for 5 days formed spontaneously beating cell monolayers and expressed regularly rhythmic action potentials and contractions (Fig. 1A to D, control panels). In this preparation, this automaticity is governed by few persisting true pacemaker cells, displaying typical diastolic depolarization (not shown), whereas the large majority of cells under record are driven cardiomyocytes (Athias and Grynberg, 1987). The averaged values of cardiomyocyte action potential and contraction parameters are given as control data in Figs. 2, 4 and 5. In this study, the cultured cardiomyocytes thus displayed cell functions that were very similar to those typically described in this cellular model (Fantini et al., 1990; Tissier et al., 2002).

3.2. Basal effects of cyclosporin A

The effects of cyclosporin A in basal conditions were evaluated by exposing cardiomyocytes to 100 and 1000 ng/ml of cyclosporin A during 24 and 48 h (Figs. 1A and B and 2). At either concentration of the drug in the incubation medium, the morphological appearance of cardiomyocytes was not altered as evaluated by microscopic examination (not shown). At the end of these incubation periods, the electrical and contractile parameters of cardiomyocytes were recorded and compared to those from control cardiomyocytes incubated during the same periods of time without drug (Figs. 1A and B and 2).

When cardiomyocytes were exposed to cyclosporin A at the lower concentration (100 ng/ml) during 24 h (Fig. 2, left column), the action potential amplitude (AP; Fig. 2B) tended to increase. On the opposite, the action potential amplitude was only slightly decreased after 48 h exposure (Fig. 2B). The action potential duration (Fig. 2B) and the action potential rate (Fig. 2C) were also subjected to slight non-significant changes after 24 h and 48 h in the presence of cyclosporin A. Moreover, cyclosporin A (100 ng/ml) had no effect on the contraction duration

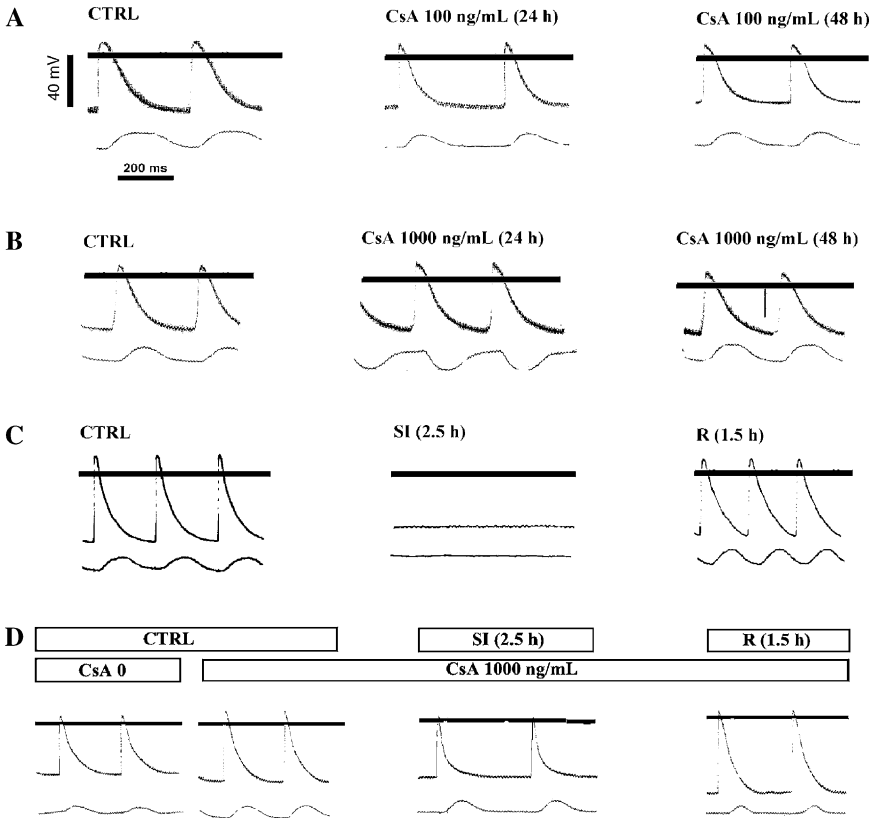

Fig. 1. (A, B) Typical recordings of action potentials (upper traces) and contractions (lower traces) of rat cardiomyocytes (CM) before (CTRL) and after sustain exposure to cyclosporin A (CsA). A, 100 ng/ml CsA; B, 1000 ng/ml CsA. (C, D) Effect of cyclosporin A of the changes in action potentials (upper traces) and contractions (lower traces) of rat cardiomyocytes induced by simulated ischemia–reperfusion. C, untreated cells; D, before (0 CsA) and after cyclosporin A addition (CsA 100, 100 ng/ml; static bath condition). Ctrl, normoxia; SI, simulated ischemia; R, reoxygenation. In each row, the recordings were obtained from the same culture dish. Scale for contraction is arbitrary. In all panels, horizontal baseline indicates zero potential level.

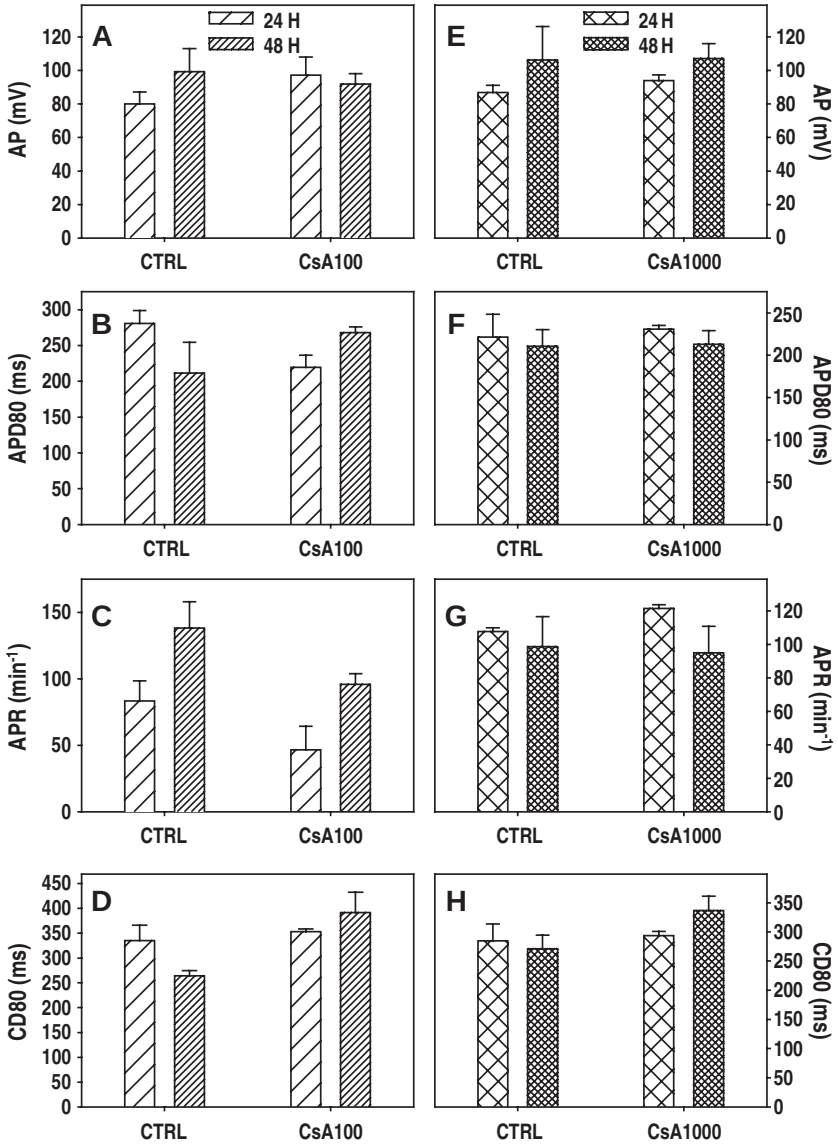

Fig. 2. Effects of chronic exposure to cyclosporin A at 100 ng/ml and 1000 ng/ml (CsA1000) during 24 h and 48 h on cardiomyocyte functions in standard culture media. CTRL: same durations of incubation without drug. A, E: action potential amplitude (AP); B, F: duration of the action potential at 80% of repolarization (APD80); C, G: frequency (APR); D, H: duration contraction in 80% of relaxation (CD80). Values represent means±S.E.M. (n=4). The differences vs. control are considered as significant at P<0.05.

(Fig. 2D) after 24 h incubation, whereas a moderate and non-significant lengthening of the contraction was seen after 48 h (Fig. 2D).

After 24 h incubation at the highest concentration (1000 ng/ml; Fig. 2, right column), cyclosporin A had no significant effect on the action potential and contraction characteristics (Fig. 2E to G), apart a moderate and non-significant increase in the spontaneous rate (Fig. 2G). After 48 h incubation, cyclosporin A at this maximal concentration did not affect the amplitude, the duration and the rate of action potentials (Fig. 2E to G). The slight change in the contraction duration (Fig. 2H) was also not significant.

In summary, the long-term presence of cyclosporin A in the growth medium did not exert cytotoxic effect on isolated cardiomyocytes on the basis of microscopic examination. Furthermore, at the tested concentrations, this chronic exposure to cyclosporin A did not alter the basal electro-mechanical functions of these cells.

3.3. Simulated ischemia–reoxygenation

As described in the Materials and methods section, The absence of substrate in the experimental medium associated to a flow of nitrogen (N_2) in the gas-controlled chamber during 2.5 h simulated in vitro the conditions of ischemia (Chevalier et al., 1990; Fantini et al., 1987; Tissier et al., 2002). The reoxygenation was achieved by the restoration of an air flow in the chamber.

The changes in electromechanical parameters during normoxia (N) followed by 2.5 h simulated ischemia (H1

to H5) and by 1.5 h reoxygenation (R1 to R3) were illustrated in Figs. 1C and 3. Simulated ischemia caused a progressive abortion of the electric activity (Figs. 1C, SI, and 3A to C) and a decrease in the resting potential (Fig. 3A, inset). The reoxygenation entailed a rapid resumption of spontaneous action potentials and contractions. After reoxygenation, the resting potential recovered a value of −70.4±4.8 mV, which was not significantly different from that measured in normoxia (−84.4±2.5 mV) (Fig. 3A, inset). Also, the values of action potential amplitude (Fig. 3A), of contraction duration (Fig. 3D) and of shortening time (Fig. 3E) did not significantly differ, at the end of reoxygenation, from values collected in control normoxia. Only the action potential duration (Fig. 3B) and the action potential rate (Fig. 3C) remained slightly different from values measured in normoxia. It was in this model of reversible ischemia–reperfusion (Fantini et al., 1987) that the effects of cyclosporin A were investigated.

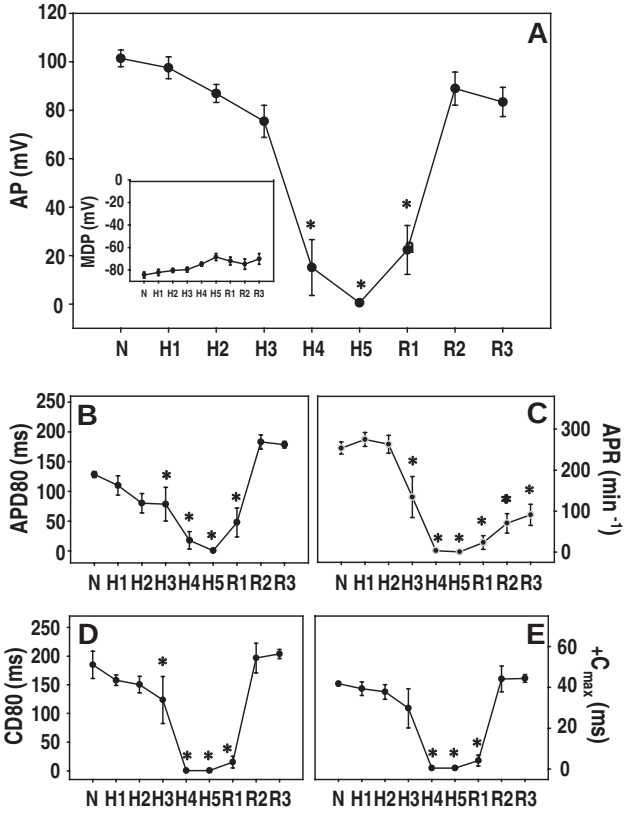

Fig. 3. Effects of simulated ischemia (SI) on cardiomyocyte functions. N: control normoxia; H1, H2, H3, H4, H5: 0.5 h, 1.5, 2 h and 2.5 h SI, respectively; R1, R2, R3: 0.5, 1, 1.5 h of reoxygenation, respectively. A, action potential amplitude (AP) and maximal diastolic potential (MDP, inset); B, duration of the action potential at 80% of repolarization (APD80); C, action potential frequency (APR); D, duration contraction in 80% of relaxation (CD80); E, shortening time ($+C_{max}$). Values represent means± S.E.M. (n=4). The differences vs. control are considered as significant at P<0.05. *, significantly different from normoxia (N).

3.4. Effects of cyclosporin A during simulated ischemia–reperfusion

Cyclosporin A at 100 ng/ml or at 1000 ng/ml was added in the experimental bath after a 1 h control period in normoxia and before the sequence of simulated ischemia followed by reoxygenation. The experimental bath was not renewed thereafter. Typical action potential and contraction recordings are shown in Fig. 1D (CsA, 1000 ng/ml). The data depicting the changes in electromechanical parameters during these protocols are given in Fig. 4 (CsA 100 ng/ml) and 5 (CsA 1000 ng/ml). During the preliminary normoxic period, cyclosporin A at 100 ng/ml as well as at 1000 ng/ml had no effects on the cardiomyocyte functions, which indicated that the acutely added immunosuppressive drug did not alter the electromechanical parameters, as observed after the long-term chronic treatments.

During simulated ischemia, cyclosporin A at 100 ng/ml did not impede the partial reduction in the resting potential observed in the absence of the drug (Fig. 4A). Conversely, cyclosporin A at this low concentration significantly reduced the simulated ischemia-induced decrease in action potential amplitude (Fig. 4B), in action potential duration (Fig. 4C) and in action potential rate (Fig. 4D). Moreover, the spontaneous cardiomyocyte contractions persisted during the whole period of simulated ischemia in the presence of 100 ng/ml cyclosporin A, whereas simulated ischemia rapidly abolished this cardiomyocyte contractile function in absence of the drug (Fig. 4E and F). Finally, cyclosporin A (100 ng/ml) did not hamper the recovery of the electrical and contractile functions of cardiomyocytes during reoxygenation (Fig. 4, A to F, R).

As observed with the lowest concentration, cyclosporin A at 1000 ng/ml had no influence on the simulated ischemia-induced progressive reduction of resting potential (Fig. 5A). On the contrary to drug-free simulated ischemia, the electrical activity was maintained during simulated ischemia (Fig. 5B to D), although a decrease in action potential amplitude (AP; Fig. 5B) and in action potential duration and rate (Fig. 5C and D, respectively) was still observed. Similarly, the contractile activity was not abolished during simulated ischemia (Fig. 5D and E). The presence of cyclosporin A during post-"ischemic" reoxygenation had no harmful effects on cells and permitted a complete recovery of the electrical activity (Fig. 5A to D, R). The same observation was done about the contractile activity (Fig. 5D and E, R). Nonetheless, cyclosporin A tended to improve the restoration of the resting potential and the action potential amplitude, since these parameters retrieved values closer to those obtained during the pre-"ischemic" control period in comparison with the electrical recovery displayed by the untreated cardiomyocytes (Fig. 5A and B, R).

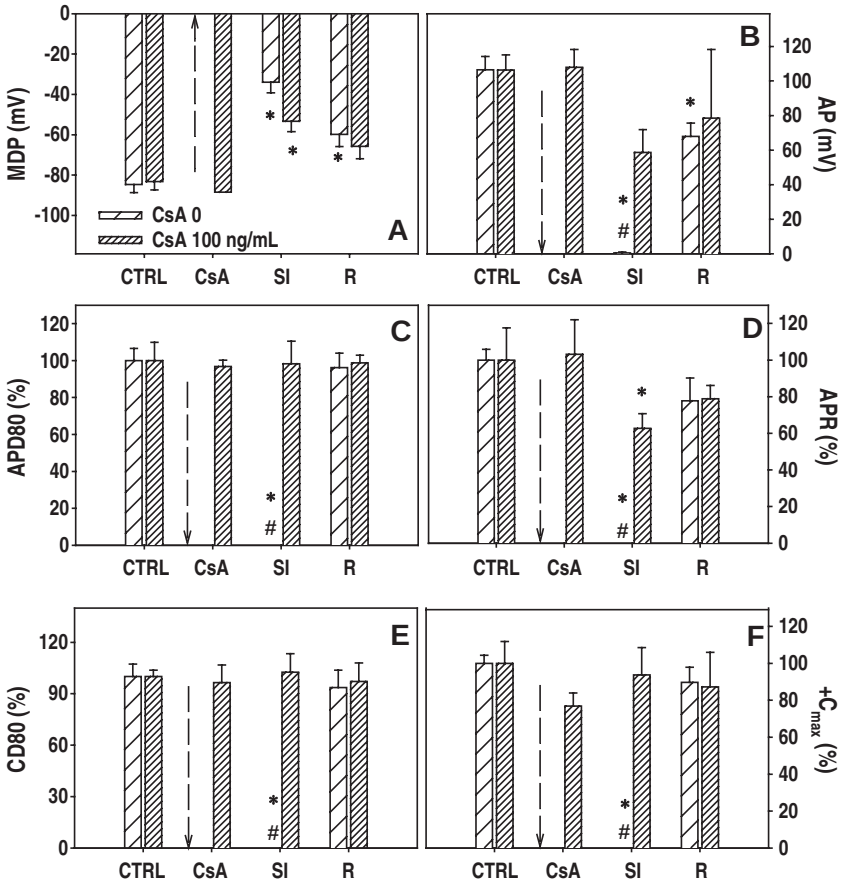

Fig. 4. Effects of cyclosporin A (100 ng/ml; CsA100) pretreatment on the cardiomyocyte dysfunctions induced by simulated ischemia (SI). CTRL, 1 h control normoxia; CsA100, 1 h cyclosporin A at 100 ng/ml in normoxia; SI, 2.5 h simulated ischemia; R, 1.5 h of reoxygenation. A, maximal diastolic potential (MDP); B, amplitude of action potential (AP); C, duration of the action potential at 80% of repolarization (APD80); D, frequency (APR); E, duration contraction in 80% of relaxation (CD80); F, shortening time ($+C_{max}$). Values represent means±S.E.M. (n=4). The differences vs. control are considered as significant at $P<0.05$. *, significantly different from normoxia (N); #, significantly different from the same situation without drug.

3.5. Effects of cyclosporin A on stress markers

3.5.1. Cell injury

The extent of simulated ischemia-induced cellular damage was evaluated by measuring lactate dehydrogenase (LDH) activity and troponine I concentration in the external bath (Fig. 6). In control normoxia, the mean (±S.E.M.) basal release in LDH was 4.30±1.14% of total LDH activity, whereas the released troponin I was undetectable. After 150 min of simulated ischemia in the absence of cyclosporin A, mean (±S.E.M.) activity of released LDH augmented up to 55.1±1.9% of total activity (Fig. 6, A). In the same conditions, troponin I concentration reached 4.0±0.3 ng/ml in the experimental bath. In the presence of cyclosporin A, the amounts of simulated ischemia-induced LDH and troponin I losses from the "ischemic" cardiomyocytes were decreased by 40% and 67%, respectively (Fig. 6, A), indicating a potent cytoprotective action of cyclosporin A.

3.5.2. Mitochondrial function

The reduction of (3,4-5 dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT) into formazan pigment was performed to assess the effect of cyclosporin A on the cardiomyocyte viability. Since this reduction predominantly relies on mitochondrial reductase activity, the MTT assay has been shown to correlate well with the mitochondrial metabolic capacity (Gomez et al., 1997).

The MTT assay was performed in the same protocols than those designed for physiological experiments. A first set of experiments assessed the effects of chronic cyclosporin A exposure (48 h) in basal growth conditions, i.e., in standard culture medium. The results showed that there was no difference between cardiomyocytes in the absence of cyclosporin A (OD_{570nm}: 0.26) and cells exposed to the drug during 48 h (OD_{570nm}: 0.242).

A second set of short-term experiments was carried out in Puck G^- medium to evaluate the influence of cyclosporin A on the simulated ischemia-induced changes of cell respiration (Table 1). Control cardiomyocytes were incubated in the normoxic glucose-free Puck's F medium and were considered as 100%. The MTT assay was performed in cardiomyocytes (i) after 1 h exposure cyclosporin A, (ii) after 1 h normoxia and 2.5 h simulated ischemia in the presence of cyclosporin A, and (iii) after 1 h normoxia, 2.5 h

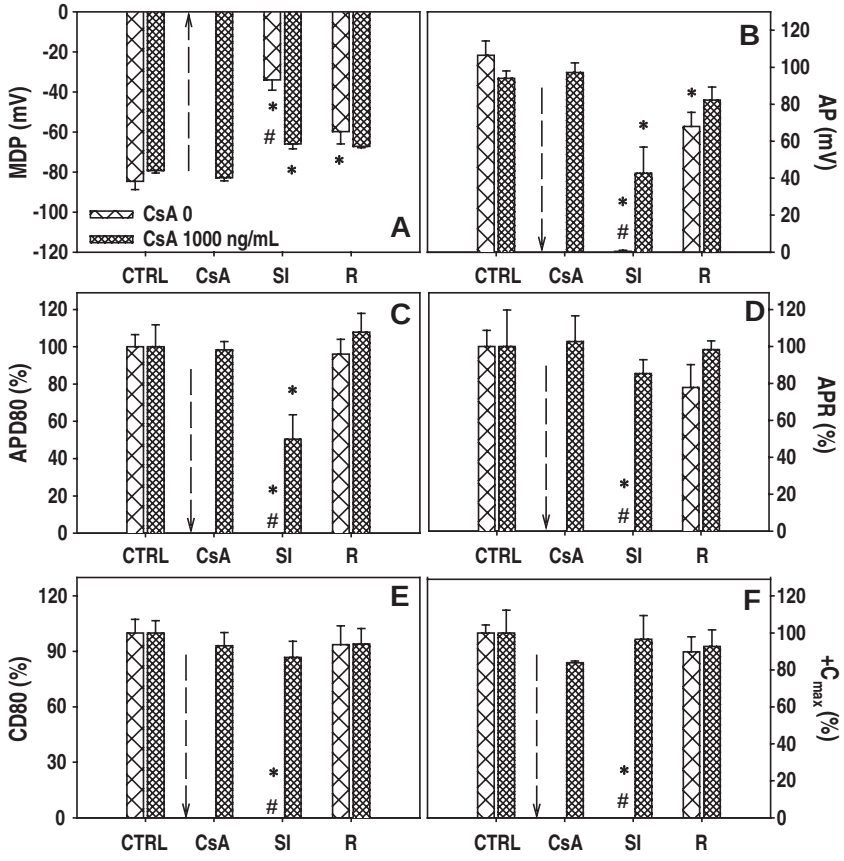

Fig. 5. Effects of cyclosporin A (1000 ng/ml; CsA1000) pretreatment on the cardiomyocyte dysfunctions induced by simulated ischemia (SI) on functions. CTRL, 1 h control normoxia; CsA1000, 1 h cyclosporin A at 1000 ng/ml in normoxia; SI, 2.5 h simulated ischemia; R, 1.5 h of reoxygenation. A, maximal diastolic potential (MDP); B, action potential amplitude (AP); C, duration of the action potential at 80% of repolarization (APD80); D, action potential frequency (APR); E, duration contraction in 80% of relaxation (CD80); F, shortening time ($+C_{max}$). Values represent means±S.E.M. (n=4). The differences vs. control are considered as significant at $P<0.05$. *, significantly different from normoxia (N); #, significantly different from the same situation without drug.

simulated ischemia and 1 h reoxygenation in the presence of the drug. When cardiomyocytes were exposed to cyclosporin A (1000 ng/ml) in normoxia, the cardiomyocyte metabolic capacity increased in comparison to the control. The 2.5 h simulated ischemia period alone (Table 1, CsA 0, 2.5 h SI) induced a strong decrease in cardiomyocyte viability. This simulated ischemia-induced fall in mitochondrial function was moderately reduced when cardiomyocytes were treated 1 h with cyclosporin A before simulated ischemia (Table 1, 1 h CsA+2.5 h SI). In untreated CM, post-"ischemic" reoxygenation failed to improve mitochondrial viability (Table 1, CsA 0, 2.5 h SI, 1.5 h R). In turn, reoxygenation following simulated ischemia in the presence of cyclosporin A (Table 1, 1 h CsA+2.5 h simulated ischemia+1 h R) restored the cardiomyocyte mitochondrial function, although the level of cell respiration reached at the end of the protocol remained below that measured in the control normoxic conditions.

3.5.3. Stress protein

Fig. 6B displays the changes in HSP70i mRNA after simulated ischemia–reperfusion challenge. As previously described, HSP70i mRNA was increased at the end of 150 min simulated ischemia (Fig. 6B, H5) (Laubriet et al., 2001). In addition, reoxygenating the "ischemic" cardiomyocytes for 90 min induced a further increase (Fig. 6B, R3). The addition of cyclosporin A had no effect on the basal level in HSP70i mRNA (Fig. 6B, CTRL). Conversely, the presence of cyclosporin A induced a significant decrease in the rises in HSP70i mRNA observed at the end of simulated ischemia and reoxygenation (−45% and −61%, respectively; Fig. 6B, H5 and R3). This inhibitory effect of cyclosporin A on the cardiomyocyte stress signaling was an additional clue on the beneficial influence of the drug.

4. Discussion

The aim of the work was to examine the specific, direct influence of cyclosporin A on the cardiac muscular cell in physiologic conditions and in an in vitro model of myocardial ischemia–reperfusion. We showed that cyclosporin A, at 100 and 1000 ng/ml, had no adverse effect on the isolated cardiomyocytes in basal conditions. Moreover, cyclosporin A exerted a potent cytoprotective action on cardiomyocytes against the changes provoked in vitro by

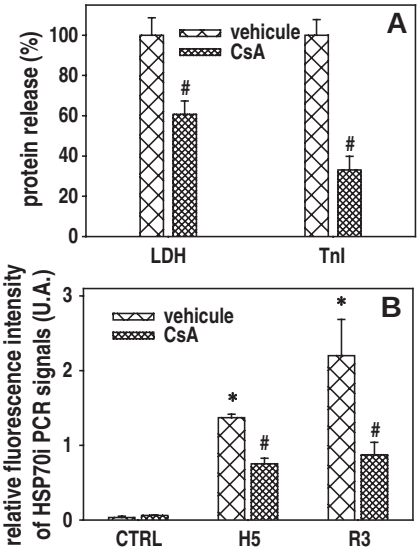

Fig. 6. (A) Effects of cyclosporin A (CsA; 1000 ng/ml) on the release of lactate dehydrogenase (LDH) and of troponine I (TnI) from cardiomyocytes after 2.5 h simulated ischemia. The protein release without drug was standardized at 100%. Basal releases in control normoxia: LDH, 4.30±1.14% of total LDH activity; troponin I, undetectable. Values are means±S.E.M. (n=12); #, significantly different from the same situation without drug. (B) Effects of cyclosporin A (1000 ng/ml) on the changes in heat shock protein 70 (HSP70) mRNA from cardiomyocytes after 2.5 h of simulated ischemia (H5) and after 2.5 h of simulated ischemia followed by 1.5 h of reoxygenation (R3). UA, arbitrary unit. The differences vs. control are considered as significant at P<0.05. Values are means±S.E.M. (n=6); *, significantly different from control normoxia (CTRL); #, significantly different from the same situation without drug.

simulated ischemia–reperfusion. This beneficial influence of cyclosporin on metabolically challenged cardiomyocytes was associated with an alleviation of protein losses, of mitochondrial dysfunction and of stress protein transcript bursts.

In basal conditions, cyclosporin A at 100 and 1000 ng/ml did not alter cardiomyocyte functions during a short-term (1 h) and long-term (24 or 48 h) treatments. Consistently, Griffiths et al. (2000) reported that cyclosporin A did not alter the contractility of isolated rat cardiomyocytes. Conversely, Kolcz et al. (1999) showed that a 15 min exposure to cyclosporin A induced changes in the beating activity of chick cardiomyocytes and that a long-term treatment with the drug (24 h) induced cytoskeletal changes in these cells. Other reports indicated an effect of cyclosporin A on transmembrane Ca^{2+} flux (Mijares et al., 1997; Park et al., 1999). In the intact heart, cyclosporin A induces changes in mechanical properties and of mitochondrial and enzymatic activities (Banijamali et al., 1993; Hutcheson et al., 1995; Kingina et al., 1991; Millane et al., 1994). To summarize, the available data from the literature tend to evoke noxious effects of cyclosporin A treatment on heart or cardiomyocytes in basal conditions, which were not observed in our work in respect of functional end-points.

Rat cardiomyocytes in our substrate-free hypoxia model of ischemia–reperfusion displayed electromechanical alterations comparable to those previously described (Fantini et al., 1987; Tissier et al., 2002). Briefly, simulated ischemia caused a gradual decrease and then an arrest of spontaneous electrical and contractile activities, associated with a moderate reduction of the maximal diastolic potential. Cardiomyocyte functions resumed upon reoxygenation to values close to those obtained in basal conditions. We have previously shown that, as in the intact experimental models, these functional changes are accompanied by a decrease in ATP content, an increase in lactate production, a release of the cellular LDH and a rise in heat shock protein (HSP) and in apoptotic inducers, these changes being attenuated by the reoxygenation (Chevalier et al., 1990; Laubriet et al., 2001).

In this model, cyclosporin A (100 and 1000 ng/ml) exerted a strong protective effect against simulated ischemia-induced cardiomyocyte dysfunctions and improved post-ischemic recovery, particularly with reference to the spontaneous rate. Similarly, cyclosporin A is able to decrease anoxia- and reoxygenation-induced myocyte hypercontraction (Duchen et al., 1993; Griffiths et al., 2000). More recently, it has been reported that cyclosporin A could protect the isolated perfused rat heart submitted to an ischemia–reperfusion from reperfusion damages (DiLisa et al., 2001), although cellular morphology or functions were unexplored in this intact preparation. Otherwise, the cardiac effects of cyclosporin A treatment have been alternatively attributed to the vehicle conveying cyclosporin A (Tatou et al., 1996), which was absent in our study, although this point remains controversial (Jurado et al., 1998).

The present data confirmed that cultured cardiomyocytes subjected to simulated ischemia released important amount of LDH and troponin I, two key markers of the degradation of the subcellular structures in vitro (Chevalier et al., 1990; Li et al., 2004). Cyclosporin A was able to reduce these losses in intracellular proteins, which suggests that the drug was also efficient to alleviate the simulated ischemia-induced cellular injury. To our knowledge, this direct demonstration of the structural cytoprotection by cyclosporin A on ischemic isolated cardiomyocytes was unprecedented. However, cyclosporin A is similarly protective against cellular necrosis in the very different contexts of

Table 1
Assessment of cell viability by MTT assay from cardiomyocytes exposed to cyclosporin A (CsA, 1000 ng/ml) or submitted to simulated ischemia–reperfusion in the absence and in the presence of cyclosporin A

Protocols	Means	S.E.M
1 h CsA	130.4	18.20
CsA 0, 2.5 h SI	34	2.0
1 h CsA→2.5 h SI	47	5.7
CsA 0, 2.5 h SI→1.5 h R	26	3.6
1 h CsA→2.5 h SI→1 h R	56	9.1

The data are expressed as percentage vs. control (n=4).
Control without MMF in basal conditions was set as 100%.
Abbreviations: CsA, cyclosporin A; SI, simulated ischemia; R, reoxygenation.

adriamicyn cardiotoxicity and of liver graft transplantation (Al-Nasser, 1998; Plin et al., 2004).

As suggested by previous works, the protective effects of cyclosporin A on the heart may be related to its ability to acts on mitochondria (Crompton, 1999; DiLisa et al., 2001; Griffiths and Halestrap, 1993; Minners et al., 2000; Suleiman et al., 2001). Therefore, in order to reveal a metabolic component in the cyclosporin A effects on cardiomyocytes, we evaluated in the same model and protocols the influence of cyclosporin A alone and before a sequence of simulated ischemia–reoxygenation using the MTT assay, which reliably evaluate viable cells with active mitochondrial function (Clerk et al., 1994; Gomez et al., 1997). In the presence of cyclosporin A, we observed that the decrease in mitochondrial metabolic activity was reduced by about 38% at the end of simulated ischemia (Table 1). Also, reoxygenation in the presence of cyclosporin A led to a higher rate of cell respiration, suggesting that the drug could improve the post-"ischemic" recovery of the mitochondrial function. In the same preparation, Sentex et al. (1999) demonstrated the slowing down of mitochondrial oxidative phosphorylations in cells exposed to cyclosporin A in basal condition only.

The protective action of cyclosporin A suggested in the pathologic heart and demonstrated here in our cellular model may also be discussed with consideration of the assumed molecular mechanisms for this molecule (Matsuda and Koyasu, 2000). The capacity of cyclosporin A to inhibit calcineurin may lead to the modulation of intracellular Ca^{2+}-dependent processes. By the same way of calcineurin inhibition, cyclosporin A may inhibit the mitochondrial permeability transition pore (mPT) (Crompton, 1999; Ly et al., 2003; Suleiman et al., 2001) and thus limit the loss of mitochondrial Ca^{2+} (Duchen et al., 1993). Moreover, a link has been suggested between calcineurin and the ryanodine receptors controlling the release of Ca^{2+} towards the cytosol (Bandyopadhay et al., 2000). Therefore, the assumed mechanisms of action of cyclosporin A data suggest that cyclosporin A could play its protective role on the cardiac muscle cells through either the limitation of cytosolic Ca^{2+} overload or the interaction with the mitochondria, although the present results relative to MTT assays seemed to favor the idea of a mitochondrial action.

According recent data, cyclosporin A could also be beneficial against tissue injury through HSP70 regulation (Chen et al., 2002). Many reports have shown an accumulation of the mRNA coding for the inducible HSP70 (HSP70i) in conditions of cellular stress (Bruce et al., 1993; Wallen et al., 1997) and have emphasized the value of this rise in HSP70i transcripts as a molecular marker of cellular response to stress (Kregel, 2002). In agreement with our previous observations (Laubriet et al., 2001), we found in cultured cardiomyocytes that simulated ischemia induced an important increase in HSP70i transcripts, which was additionally augmented by the reoxygenation simulation of reperfusion. Cyclosporin A decreased both these simulated ischemia- and reoxygenation-induced accumulation in HSP70i transcripts. Oppositely, Chen et al. (2002) have suggested that cyclosporin A could protect against oxidative stress-induced cardiomyocyte apoptosis at least partly through HSP70 up-regulation. This was not the case in our conditions, suggesting that the ischemia–reperfusion stress signal perceived by the myocardial cells was attenuated in presence of cyclosporin A. This depressing influence of cyclosporin A on stress signaling pathway in the "ischemic-reperfused" in vitro cardiomyocytes resembled the cyclosporin A-induced down-regulation of stress protein in chronic inflammation (Schett et al., 1998). Similarly, it has been recently demonstrated that cyclosporin A inhibits cellular adaptative response mediated by hypoxia-inducible factor 1α (HIF-1α) (Dangelo et al., 2003).

To summarize, the present study provide the first integrative account about the beneficial effect of cyclosporin A upon the functional, biochemical and ultrastructural damages of the ischemic myocardial cells. These results shed a new light on the protective effect of cyclosporin A, which might involve not only the limitation of lymphocyte T activation but also a direct protective action on physiology, metabolism, ultrastructure and stress signaling of cardiomyocytes exposed to an ischemia–reperfusion insult. Tacking into consideration the differing data about the cyclosporin A mechanisms of action, it seems relevant to try to clarify the cellular and molecular ways by which cyclosporin A protects the cardiac muscle cells. In particular, a possible early action through the cytoskeletal organization (Kolcz et al., 1999; Vandroux et al., 2004) and the HIF-1α "paradox" are currently under study in our group. Finally, the present work emphasizes the interest of the heart cell cultures for acute and chronic experimental studies in the field of the basic pharmacology of immunosuppressive drugs.

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