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Concentration dependent mitochondrial effect of amiodarone

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Abstract

Although, the antiarrhythmic effect of amiodarone is well characterized, its effect on post-ischemic heart and cardiomyocytes, as well as the mechanism of its toxicity on extracardiac tissues is still poorly understood. In this study, we analyzed energy metabolism *in situ* during ischemia-reperfusion in Langendorff-perfused heart model by measuring the high-energy phosphate metabolites using ^{31}P NMR spectroscopy. The toxicity of amiodarone on cardiomyocytes and cell lines of extracardiac origin, as well as direct effect of the drug on mitochondrial functions in isolated mitochondria was also analyzed. Amiodarone, when was present at low concentrations and predominantly in membrane bound form, protected heart and mitochondrial energy metabolism from ischemia-reperfusion-induced damages in Langendorff-perfused heart model. Toxicity of the drug was significantly higher on hepatocytes and pancreatic cells than on cardiomyocytes. In isolated mitochondria, amiodarone did not induce reactive oxygen species formation, while it affected mitochondrial permeability transition in a concentration dependent way. Up to the concentration of 10 μM, the drug considerably inhibited Ca^{2+}-induced permeability transition, while at higher concentrations it induced a cyclosporin A independent permeability transition of its own. At concentrations where it inhibited the Ca^{2+}-induced permeability transition ($IC_{50} = 3.9 \pm 0.8$ μM), it did not affect, between 6 and 30 μM it uncoupled, while, at higher concentrations it inhibited the respiratory chain. Thus, the concentration dependent nature of amiodarone's effect on permeability transition together with the different sensitivities of the tissues toward amiodarone can be involved in the beneficial cardiac and the simultaneous toxic extracardiac effects of the drug.

Keywords: Amiodarone; Langendorff-perfused heart; Mitochondria; Permeability transition; High-energy phosphates; ^{31}P NMR

1. Introduction

Amiodarone (2-butyl-3-benzofuranyl-4-[2-(diethylamino)-ethoxy]-3,5-diiodophenyl-ketone hydrochloride), a class III antiarrhythmic agent prolongs action potential duration which effect may involve blocking of β-adrenergic receptors, sodium channels and L-type calcium channels [1–4]. It is one of the most effective antiarrhythmic drugs, and is frequently used in the clinical practice. However, its administration is often limited by its toxic side effects, including pulmonary, liver and pancreas fibrosis, and thyroid dysfunction [5–7]. The beneficial antiarrhythmic effect and the toxic effects on extracardiac tissues indicate a different sensitivity of the tissues toward amiodarone.

Conflicting reports appeared about the effect of the drug on cardiac functions in human studies [8,9]. Some reports presented beneficial effects of amiodarone during ischemia-reperfusion of perfused rat hearts [10,11], whereas according to another study, the drug improved hemodynamic parameters while it worsened the damages to the mitochondrial energy metabolism caused by ischemia-reperfusion [7]. The various results indicate that the molecular mechanism of the drug on cardiomyocyte function is poorly understood.

The effect of amiodarone on mitochondrial functions is also contradictory. Some data indicate that it has inhibitory effect on respiratory complexes I and II [12,13], as well as on the activity of F_0F_1ATPase [14], whereas other reports emphasize its protective effect on mitochondrial functions

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Abbreviations: Δψ, mitochondrial membrane potential; CsA, cyclosporin A; ROS, reactive oxygen species; PTP, permeability transition pore; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

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[15]. Amiodarone was also shown to inhibit the tricarboxylic acid cycle, the beta oxidation of fatty acids, and carnitine palmitoyl transferase 1 [16].

Up to now, no reports appeared about the effect of amiodarone on mitochondrial permeability transition, although the latter plays a major role in mediating both apoptotic and necrotic cell death [17,18]. Various stimuli, including elevated intracellular Ca^{2+} concentration, induce the opening of a megachannel in the inner mitochondrial membrane leading to equilibration of the ions within the intermembrane space (and thus the cytosol) and the mitochondrial matrix, dissipation of the $\Delta\psi$ and uncoupling of the respiratory chain. Numerous agents, including CsA, can prevent the opening of the PTP. The volume disregulation following the opening of the PTP causes swelling of the matrix, leading to membrane disruption and finally necrotic cell death [18]. Dissipation of $\Delta\psi$ induces the release of mitochondrial proteins like cytochrome c and apoptosis inducing factor (AIF) resulting in activation of the caspase-cascade, and ultimately apoptotic cell death [17,18].

In this study, we determined the effect of amiodarone on mitochondrial energy-metabolism during ischemia-reperfusion of Langendorff-perfused rat hearts by *in situ* ^{31}P NMR spectroscopy. Assessing the direct effect of the drug on cardiac and extracardiac tissue, we determined the cytotoxicity of amiodarone on cardiomyocytes and hepatocytes in culture. In an attempt to reveal the underlying mechanism of the drug in these paradigms, we studied its direct mitochondrial effects including induction of reactive oxygen species production, opening of PTP, and its effect on the mitochondrial respiration.

2. Materials and methods

2.1. Chemicals

CsA was from Biomol Research Labs. Inc.; Dihydrorhodamine123 (DRh123) were from Molecular Probes; all other compounds were from Sigma Chemical Co. unless otherwise stated.

2.2. Animals

Wistar rats were purchased from Charles River Hungary Breeding Ltd. The animals were kept under standardized conditions; tap water and rat chow were provided *ad libitum*. Animals were treated in compliance with approved institutional animal care guidelines.

2.3. Cell culture

PANC-1 human pancreatic epithelioid carcinoma cells, BRL-3A rat liver cells, H9C2 mouse cardiomyocytes and Sp-2 mouse myeloma cells were from American Type Culture Collection. PANC-1, BRL-3A, and H9C2 cells were cultured in DMEM containing 1% antibiotic-antimycotic solution (Sigma) and 10% FCS. Sp-2 cells were cultured in RPMI 1640 medium containing L-glutamin (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FSC. Cells were passaged at intervals of 3 days.

2.4. Heart perfusion

Pre-treatment with amiodarone was done exactly as described previously [15]. Hearts were perfused via the aorta as described before [19]. After a washout (non-recirculating period of 15 min), hearts were perfused under normoxic conditions for 10 min; the flow was subsequently discontinued for 30 min by inflating a balloon (ischemia), which was followed by 15 min of reperfusion. Levels of high-energy phosphate intermediates were monitored in the magnet of a ^{31}P NMR spectroscope during the entire perfusion.

2.5. NMR spectroscopy

NMR spectra were recorded with a Varian ^{UNITY}INOVA 400 WB instrument as described [32]. Data were acquired from five independent experiment for sham-operated and amiodarone-treated groups each.

2.6. Cell viability assay

PANC-1, BRL-3A, and H9C2 cells were seeded into 96-well plates at a starting density of 2.5×10^4 cell/well and cultured overnight. The following day, amiodarone at the indicated concentrations was added to the medium. Forty-eight hours later, 0.5% of the water soluble mitochondrial dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT^+) was added. Incubation was continued for 3 more hours, the medium was removed, and the water insoluble blue formasan dye formed stochiometrically from MTT^+ was solubilized by acidic isopropanol. Optical densities were determined by an ELISA reader (Anthos Labtech 2010) at 550 nm wavelength. All experiments were run in at least four parallels and repeated three times.

2.7. DNA electrophoresis

Sp-2 and H9C2 cells at a starting density of 6×10^6/10 cm plate were cultured in the presence of different concentrations of amiodarone for 24 hr, then were collected. Cell viability was assessed by the trypan blue dye exclusion method. DNA fragmentation was monitored by gel electrophoresis as described before [20]. Electrophoresis was performed and DNA was visualized under UV light and photographed. Data presented as representative photomicrographs of at least three parallel experiments.

2.8. Isolation of mitochondria

Liver and heart mitochondria were prepared according to standard protocol [21]. Only difference among the organs was in the primary homogenization protocol; liver was squeezed through a liver press, while pooled heart tissue from five rats was minced with a blender. All isolated mitochondria were purified by Percoll gradient centrifuging [22].

Mitochondrial oxygen consumption was assessed by a Clark electrode. The mitochondrial respiration was measured in the presence of different concentrations of amiodarone. Before performing experiments, respiratory control ratio (RCR) values of the mitochondria were determined and were found to be in the range of 2.1 ± 0.4 and 3.7 ± 0.6 for the mitochondria from the heart or liver respectively [23,24]. States 3 and 4 respiration as well as the respiratory control ratio and the ADP/O ratio were assessed from the original registration curve.

2.9. Oxygen consumption of cell lines

H9C2, PANC-1, and BRL-3A were assessed by a Clark electrode [25,26]. The cells (1.5×10^7 cells/mL) were permeabilized by 50 μg/mL digitonin to enable the penetration of the substrates and the different concentrations amiodarone.

2.10. Mitochondrial permeability transition

Mitochondrial permeability transition was monitored by following the accompanying large amplitude swelling via the decrease in absorbance at 540 nm [27] measured by a Perkin-Elmer fluorimeter in reflectance mode. Mitochondrial permeability transition was induced by the addition of 60 μM Ca^{2+} or of amiodarone at the indicated. Decrease of E_{540} and fluorescence intensity changes were detected for 20 min. The results are demonstrated by representative original registration curves from at least five independent experiments, each repeated three times using mitochondria prepared from the same liver or pool of rat hearts, respectively.

2.11. Enzymatic permeability transition assay

Measuring of citrate synthase (EC 4.1.3.7) and carnitine acyl transferase (EC 2.3.1.7) activity after the induction of permeability transition was performed as described [28,29].

2.12. The determination of ROS formation

ROS formation detected [29] the fluorescence of Rh123 formed by ROS-induced oxidation of the non-fluorescent DRh123 *in situ* at an excitation wavelength of 495 nm and an emission wavelength of 535 nm by a Perkin-Elmer fluorimeter. The ROS-induced oxidation of *N*-acetyl-8-dodecyl-3,7-dihydroxyphenoxazine forms *N*-acetyl-8-dodecyl-resorufin (resorufin) which exhibits strong red fluorescence. This product is well retained in living cells and organelles by virtue of its lipophilic tail, making it possible to detect ROS production in the lipid phase. The method is the same as described above except for changing the excitation wavelength to 578 nm and the emission wavelength to 597 nm. ROS formation was calculated from the slope of the registration curves.

2.13. Statistical analysis

Data were presented as means ± SEM. For multiple comparison of groups, ANOVA was used. Statistical difference between groups was established by paired or unpaired Student's *t*-test, with Bonferroni correction.

3. Results

3.1. Effect of amiodarone on the energy metabolism during ischemia-reperfusion in perfused hearts

Concentrations of high-energy phosphate intermediates were monitored during ischemia-reperfusion in Langendorff-perfused hearts by using ^{31}P NMR spectroscopy. In order to study the effect of amiodarone on the energy-metabolism of the perfused hearts, a single i.v. injection of 20 mg/kg amiodarone was administered to a group of rats 30 min before the start of the heart-perfusion, a protocol that was previously reported to be optimal for cardioprotection by amiodarone [15]. Thirty minutes of global ischemia induced the disappearance of creatine phosphate (Fig. 1A) and ATP (Fig. 1B), and a gradual increase of inorganic phosphate (Fig. 1C) signal. During reperfusion, all hearts restarted working, while creatine phosphate concentrations in hearts of sham-operated animals recovered to 35% of their normoxic level. Amiodarone pre-treatment resulted in a significantly higher recovery of creatine phosphate concentrations during the ischemia-reperfusion cycle (74%, Fig. 1A). In addition, amiodarone pre-treatment slightly but significantly delayed the decrease of ATP concentrations during ischemia, while improved the recovery of ATP level (Fig. 1B) and facilitated the faster and more complete utilization of inorganic phosphate (Fig. 1C) during reperfusion.

3.2. Effect of amiodarone on cultured cell lines

Viability of PANC-1, BRL-3A, and H9C2 cells exposed to different concentrations of amiodarone for 48 hr was assessed by the MTT^+ method (Fig. 2A). Amiodarone, at a concentration of 30 μM and higher decreased the viability of H9C2 cardiomyocytes. Whereas, in the whole concentration range, extracardiac cell lines were more susceptible to

amiodarone toxicity, as revealed by their decreased viability data. All differences from viability data of H9C2 line was statistically significant ($P < 0.001$). BRL-3A hepatocytes were much more sensitive to amiodarone toxicity than the PANC-1 pancreatic carcinoma cells; however, BRL-3A was a normal, while PANC-1 a cancer cell line.

This cytotoxicity of amiodarone seemed to be apoptotic as it was revealed by DNA fragmentation, a widely used marker of apoptosis, in Sp-2 mouse myeloma and H9C2 cardiomyocyte cells. DNA fragmentation was visible with amiodarone concentration of 10 μM or higher in Sp-2 cells (Fig. 2B). However, in the case of H9C2 cardiomyocyte cells treated with amiodarone up to the concentration of 30 μM, no DNA fragmentation was detected (data not shown).

3.3. The effect of amiodarone on the oxygen consumption

The oxygen consumption of isolated mitochondria was measured by a Clark electrode, with 10 mM succinate as substrate in the absence (state 4 respiration) or presence of

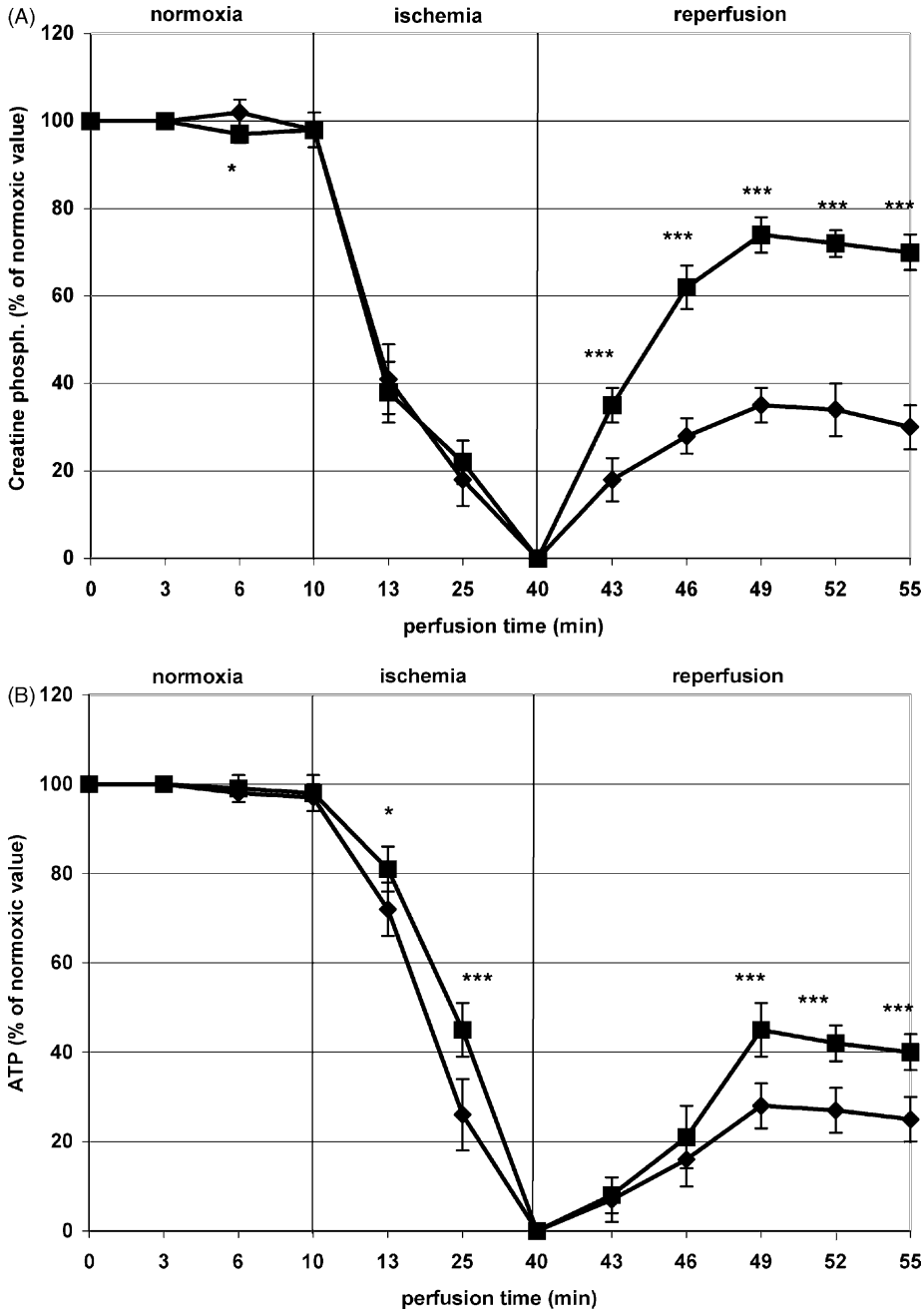

Fig. 1. The effect of amiodarone on the high-energy phosphate metabolism in Lagendorff-perfused rat hearts. Groups of five rats were treated by a single shot of physiological salt solution (sham-operated) or amiodarone 20 mg/kg i.v. 30 min before sacrifice. Their hearts were removed, applied to a Langendorff perfusion apparatus which was inserted into the magnet of an NMR spectrometer. Concentrations of creatine phosphate (A), ATP (B), and inorganic phosphate (C) were measured *in situ* by ^{31}P NMR spectroscopy in the perfused hearts subjected to 30 min of ischemia followed by 15 min of reperfusion. Data represents average ± SEM. Note that the time axis is not proportional. * ($P < 0.05$); *** ($P < 0.001$) significant differences from control.

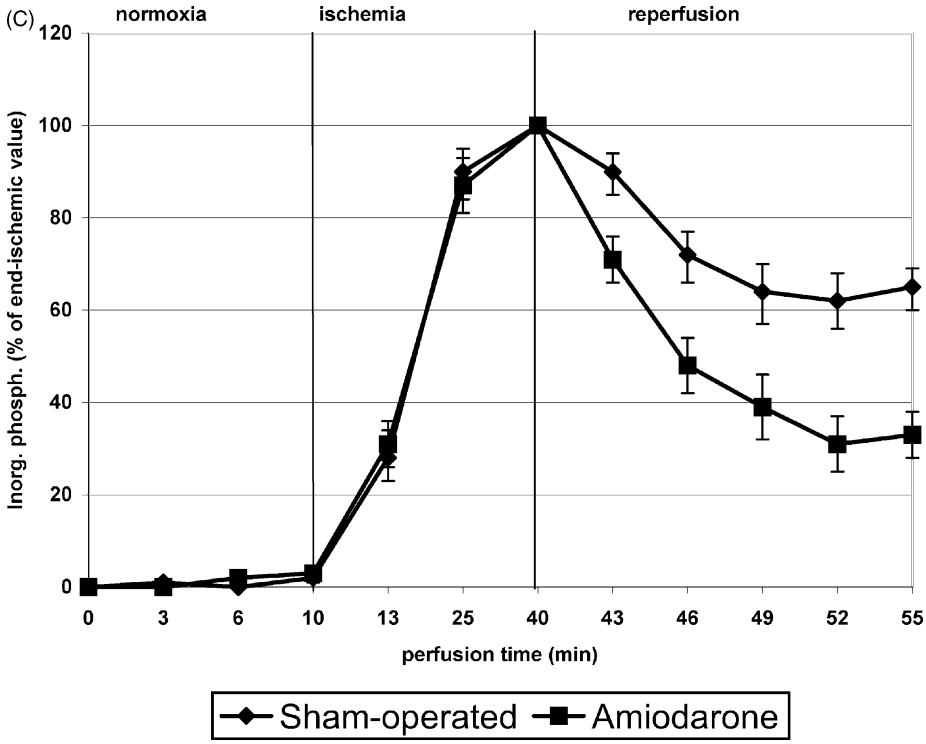

Fig. 1. (*Continued*).

3.3 mM ADP (state 3 respiration) exposed to different concentrations of amiodarone. In isolated rat heart mitochondria, at low concentrations of up to 6 μM in the case of states 3 and 4 respiration, amiodarone did not have any significant effect. In an intermediate concentration range of 6–30 μM, it increased both the states 3 and 4 oxygen consumption, while, it gradually inhibited it at higher concentrations (Fig. 3A). The respiratory control rate (RCR)—the fraction of states 3 and 4 respiration—moderately increased from it's initial value of 2.1 in the presence of amiodarone at concentrations of up to 6 μM, showing a radical decrease in the concentration range of 6–30 μM. At higher concentrations the value of RCR was leveled to 1. The ADP/O ratio showed a decrease in the presence of amiodarone up to the concentration of 30 μM. The effect of amiodarone on isolated rat liver mitochondria was basically the same (data not shown).

The respiration of PANC-1, BRL-3A, and H9C2 cells exposed to different concentrations of amiodarone was assessed by a Clark electrode. The cells (1.5×10^7 cells/mL) were permeabilized with 50 μg/mL of digitonin to enable the penetration of the substrates and the different concentrations amiodarone administered as in the case of isolated mitochondria. In H9C2 cells amiodarone increased the state 4 respiration in the presence of amiodarone up to the concentration of 30 μM, while at higher concentrations the drug presented an inhibitory effect on respiration. The state 3 respiration was not significantly altered by amiodarone up to the concentration of 10 μM, while at higher concentrations the respiration in the presence of ADP was gradually smaller (Fig. 3B). In BRL-3A cells, amiodarone increased the state 4 respiration up to the concentration of 10 μM, while at higher concentrations the oxygen consumption gradually decreased. In this cell line, amiodarone did not affect the state 3 respiration, and in the concentration of 10 μM or higher it presented an inhibitory effect (Fig. 3C). In PANC-1 cell lines, amiodarone did not increase either the states 3 or 4 in low concentrations, whereas in inhibited both in the concentration of 10 μM or higher (Fig. 3D).

3.4. Effect of amiodarone on permeability transition in isolated mitochondria

In order to demonstrate the direct effect of amiodarone on the mitochondrial permeability transition, we monitored mitochondrial swelling and opening of the PTP by enzyme assays from isolated, Percoll gradient purified rat liver and heart mitochondria. High-amplitude swelling of the mitochondria due to permeability transition was monitored by the decrease of reflectance of 540 nm light.

In isolated liver mitochondria, the swelling induced by 60 μM Ca^{2+} was completely inhibited by 2.5 μM CsA (Fig. 4A, line 3). Depending on its concentration, amiodarone had a concentration dependent effect on mitochondrial swelling. Up to the concentration of 10 μM, amiodarone inhibited the rapid swelling induced by Ca^{2+} in a concentration dependent manner (Fig. 4A, lines 4–7) with the IC_{50} of 3.9 ± 0.8 μM. However, at higher concentrations, it induced swelling by its own which developed with a rate

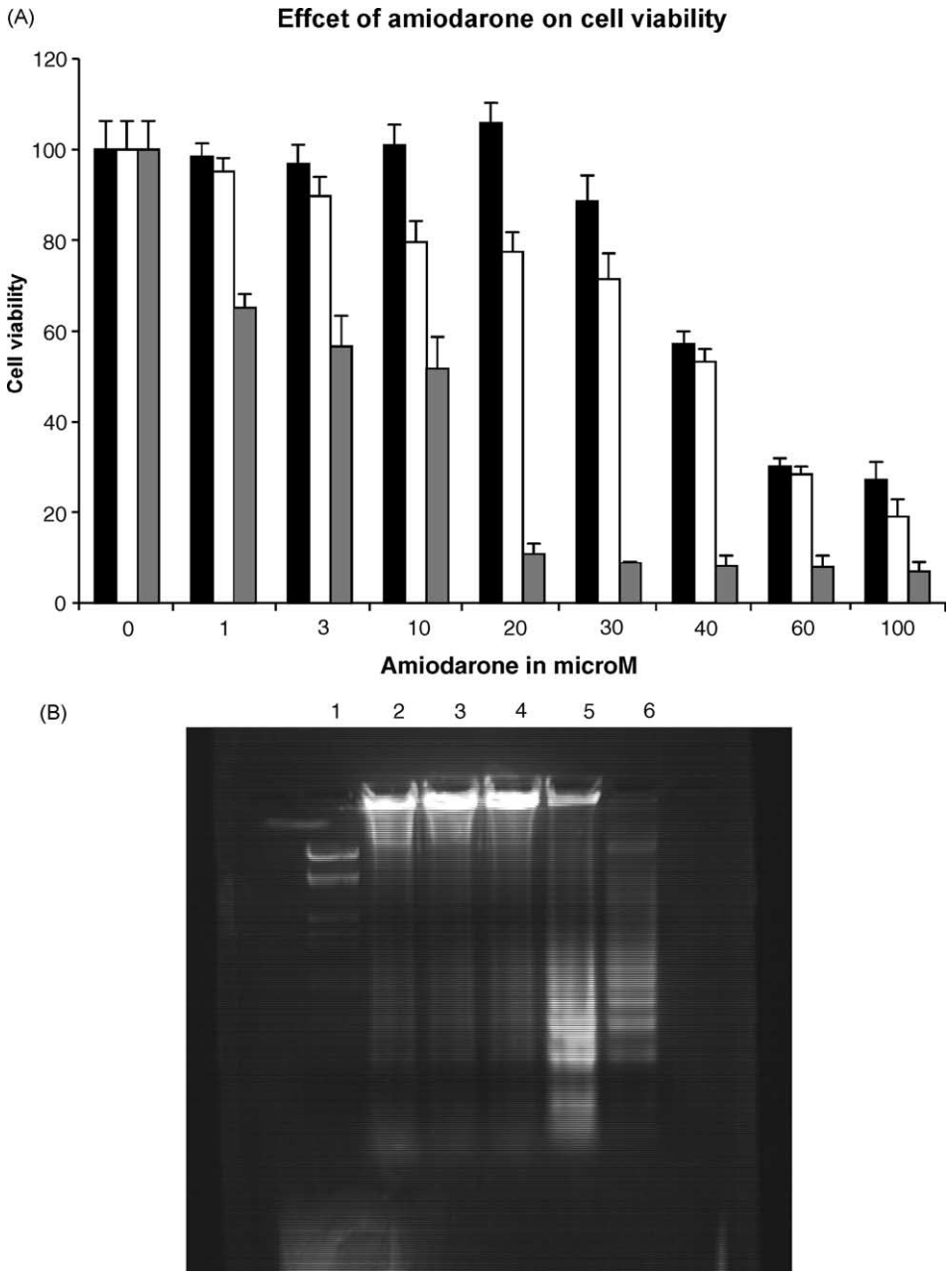

Fig. 2. The effect of amiodarone on viability of cell lines and apoptosis in SP-2 cells. The effect of amiodarone on viability (A) of H9C2 cells (filled bars), PANC-1 (open bars), and BRL-3A (striped bars) was detected by the formation of water insoluble blue formasan dye from the yellow mitochondrial dye, MTT^+ by the functionally active mitochondria of the cells. The cells were exposed to different concentrations of amiodarone for 48 hr before the addition of the MTT^+ dye. Data represents average ± SEM of three independent experiments running in four parallels. All differences from viability data of H9C2 line was statistically significant ($P < 0.001$). Amiodarone-induced DNA fragmentation analysis (B) was performed by separating the total DNA content of SP-2 cells exposed to different concentrations of Ad for 24 hr by agarose gel electrophoresis. Lane 1: marker; lane 2: no Ad; lane 3: 1 μM Ad; lane 4: 3 μM Ad; lane 5: 10 μM Ad; lane 6: 30 μM Ad.

slower than that of the Ca^{2+}-induced swelling (Fig. 5A, lines 3, 5, and 7), and was not inhibited by 2.5 μM CsA (Fig. 5A, lines 4, 6, and 8). Due to its conflicting effects, 20 μM amiodarone was less effective in delaying the Ca^{2+}-induced swelling than 10 μM (Fig. 4A, lines 7 and 8).

Amiodarone at the concentration of 10 μM that was found to be most effective in the case of liver mitochondria, inhibited the 60 μM Ca^{2+}-induced swelling in heart mitochondria about the same way as 2.5 μM CsA did (Fig. 4B, lines 3 and 6); however, amiodarone at the concentration of 20 μM proved to be less effective against the swelling induced by 60 μM Ca^{2+} (Fig. 4B, line 7). At higher concentration, amiodarone induced swelling by its own, that was not inhibited by 2.5 μM CsA (Fig. 5B, lines 5 and 6), similarly as it did in the case of liver mitochondria.

CsA not only inhibited the swelling induced by 60 μM Ca^{2+} (Fig. 4A, line 3), but it also arrested the progress of the Ca^{2+}-induced swelling when added half-way through

(Fig. 6A, blank arrow 1, line 3). In contrary to this, amiodarone when added half-way through only delayed the progress of the swelling (Fig. 6A, blank arrow 1, line 4); however, the re-onset of the swelling could be arrested by the addition of 2.5 μM CsA (Fig. 6A, blank arrow 2, line 5). Up to the concentration of 10 μM, amiodarone delayed the progress of the swelling when added before the addition of 60 μM Ca^{2+} (Fig. 4A, line 7; Fig. 6B, line 3). When the

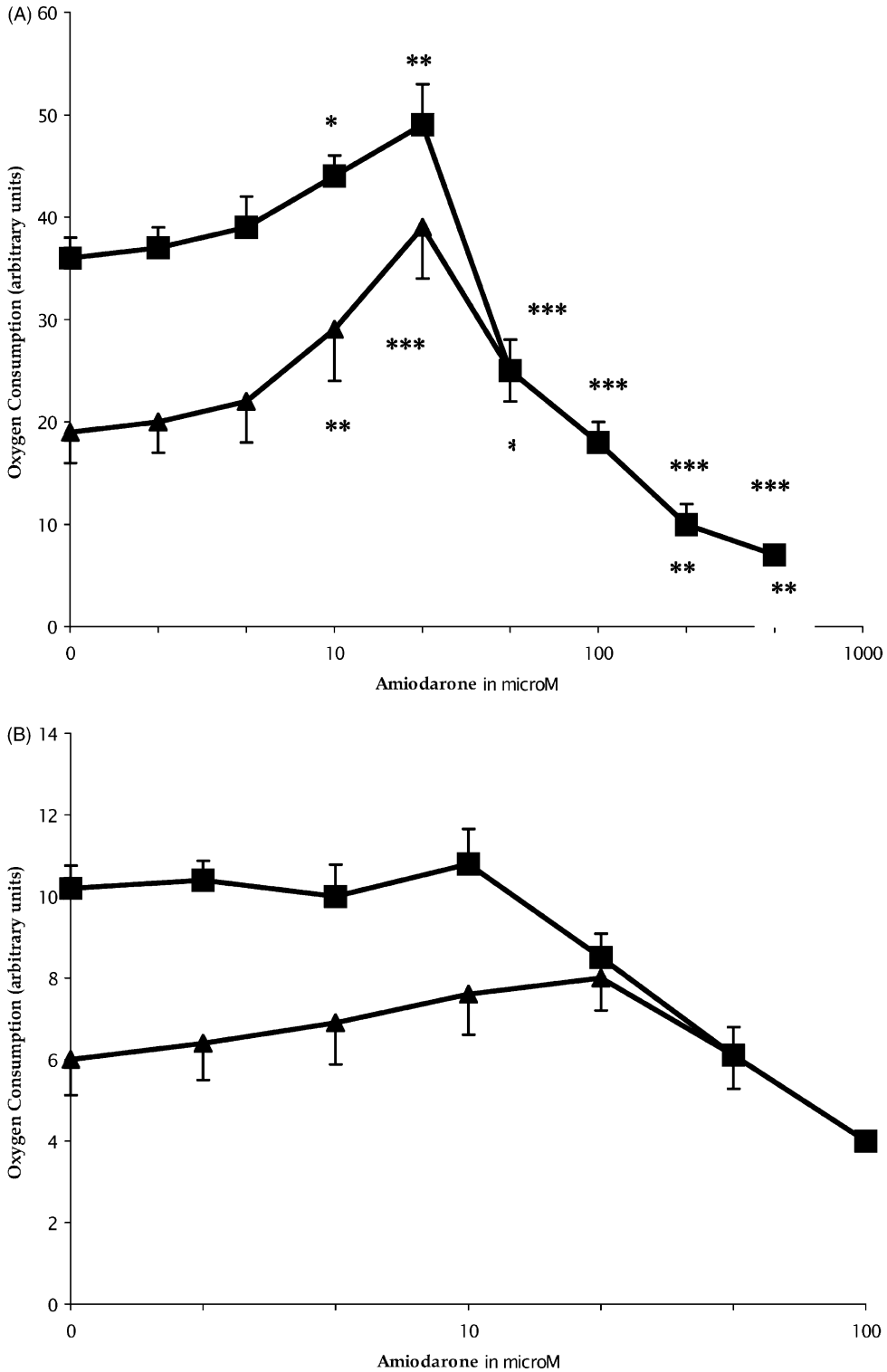

Fig. 3. The effect of amiodarone on oxygen consumption. Mitochondrial respiration was measured by a Clark electrode with 10 mM succinate as substarte (state 4 respiration) or in the presence of 3.3 mM ADP (state 3 respiration) and different concentrations of amiodarone in isolated mitochondria (A), H9C2 cardiomyocyte cells (B), BRL-3A rat liver cells (C), and PANC-1 pancreatic cells (D) permeabilized by digitonin. Data represents average ± SEM of three independent experiments repeated twice from the same samples. Note that the concentration axis is logarithmic. * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$) significant differences from control.

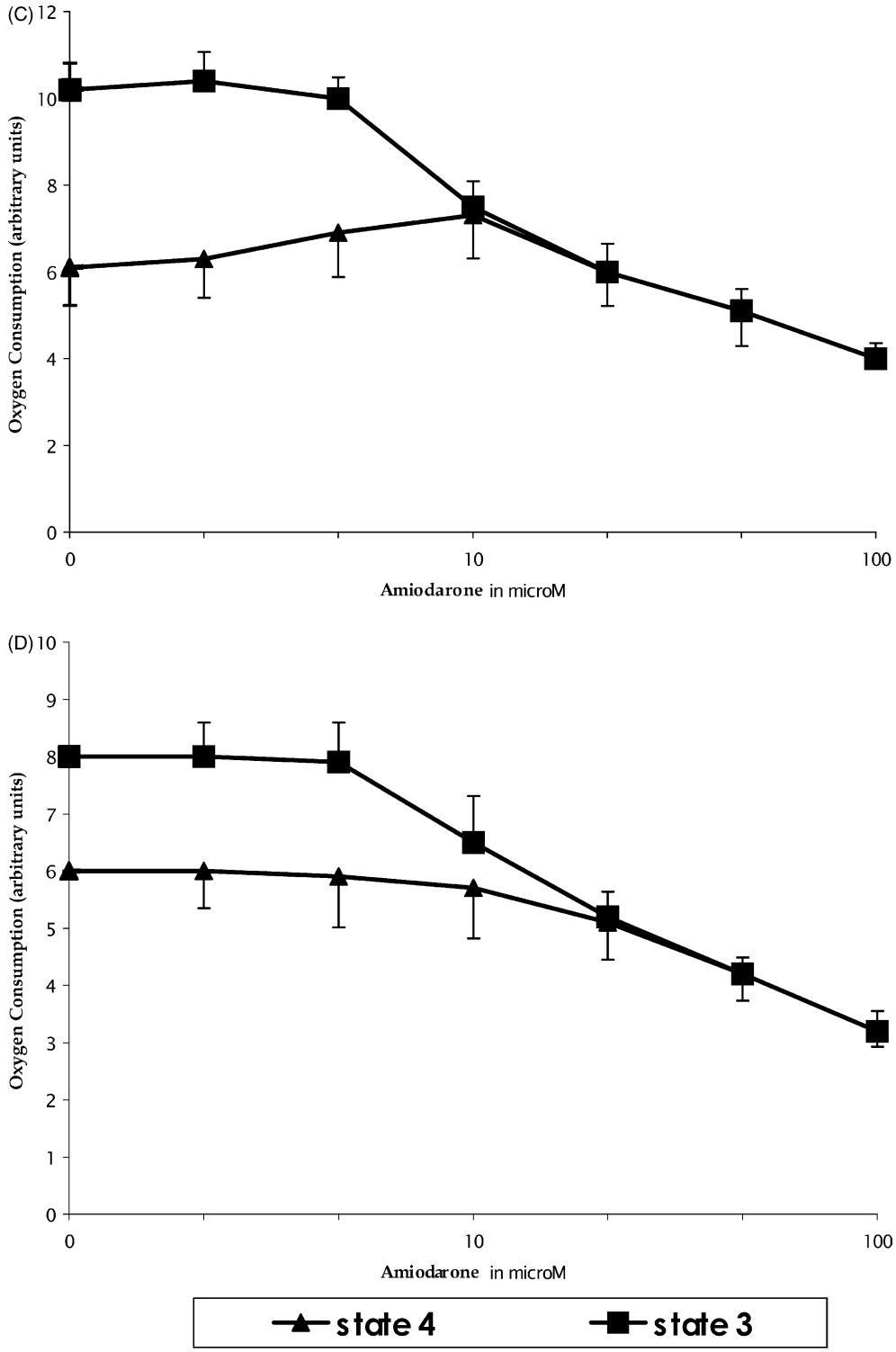

Fig. 3. (*Continued*).

same amount of amiodarone was added again half-way during the Ca^{2+}-induced swelling (Fig. 6B, blank arrow), it did not arrest the progress of the swelling; however, the addition of 2.5 μM CsA at the same time prevented the further swelling of the mitochondria (Fig. 6A, blank arrow, line 4). These indicate that CsA and amiodarone affects mitochondrial swelling by a different mechanism.

Since amiodarone was shown to have inhibitory effect on respiratory complex I [12], we analyzed the effect of rotenone, a well known inhibitor of the NADH dehydrogenase on the mitochondrial swelling. Rotenone, at the concentration of 3 μM or higher, similarly to CsA, completely inhibited the swelling induced by 60 μM Ca^{2+}, and it did not induce swelling in higher concentrations of up to

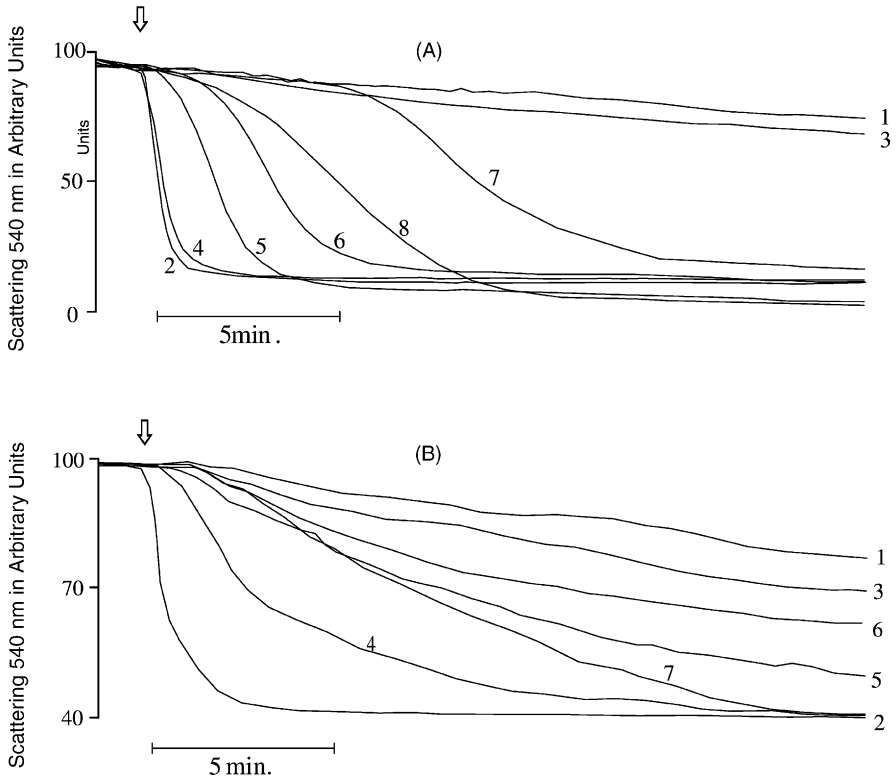

Fig. 4. The effect of amiodarone (Ad) on Ca^{2+}-induced mitochondrial swelling. Mitochondrial swelling was demonstrated by monitoring E_{540} in isolated rat liver (A) and heart (B) mitochondria. Amiodarone at the indicated concentration or 2.5 μM CsA was present throughout the experiment. Mitochondrial swelling was induced by adding 60 μM Ca^{2+} at arrow. (A) Line 1: baseline swelling (no agent); line 2: no Ad or CsA; line 3: CsA; line 4: 1 μM Ad; line 5: 2.5 μM Ad; line 6: 5 μM Ad; line 7: 10 μM Ad; line 8: 20 μM Ad. (B) Line 1: baseline swelling (no agent); line 2: no Ad or CsA; line 3: CsA; line 4: 2.5 μM Ad; line 5: 5 μM Ad; line 6: 10 μM Ad; line 7: 20 μM Ad.

50 μM (data not shown). Amiodarone also showed uncoupling effect, as supported by the results of the mitochondrial respiration described earlier. Therefore, we analyzed the effect of low concentrations of FCCP (a widely used uncoupling agent) on the swelling. FCCP at the concentration of 1 μM or higher completely inhibited the Ca^{2+}-induced mitochondrial swelling, while it did not induce swelling on its own (data not shown). This indicates that though amiodarone has both uncoupling and inhibitory effect, it's effect on mitochondrial swelling is exerted by a different mechanism.

3.5. Demonstration of permeability transition by enzymatic assay

When the inner mitochondrial membrane is intact, only substrates that are transported through the membrane can access matrix enzymes. However, opening of the PTP allows the entry of such substrates into the mitochondrial matrix. We demonstrated the Ca^{2+}-induced opening of the PTP by measuring citrate synthase and carnitine acyl transferase activities in isolated rat heart mitochondria using externally added acetyl-coenzyme A. The Ca^{2+}-induced opening of the permeability pore was inhibited by 10 μM amiodarone (Fig. 7) in a similar extent as it was inhibited by 2.5 μM CsA (data not shown).

3.6. The effect of amiodarone on mitochondrial ROS production

Since ROS formation can induce mitochondrial permeability transition, we studied the effect of amiodarone on ROS production in isolated, Percoll gradient purified rat heart and liver mitochondria. ROS formation was measured by monitoring the green or red fluorescence of Rh123 or resorufin oxidized by the ROS from non-fluorescent DRh123 or *N*-acetyl-8-dodecyl-3,7-dihydroxyphenoxazine *in situ*. By virtue of its dodecyl group, resorufin is localized in membranous regions and detect ROS formation in lipid phase while Rh123 fluorescence reflects to ROS levels in aqueous phase. Amiodarone did not induce ROS production in either case in the concentration range of 1–100 μM (data not shown).

4. Discussion

Amiodarone exerts damaging effects on extracardiac tissues such as lung, thyroid gland, liver, and pancreas [5,6,13], whereas reports on the effects of amiodarone on cardiac function have been variable. Some emphasized its beneficial effect on cardiac functions and arrhythmia following ischemia and reperfusion [7,15,30]; however, other

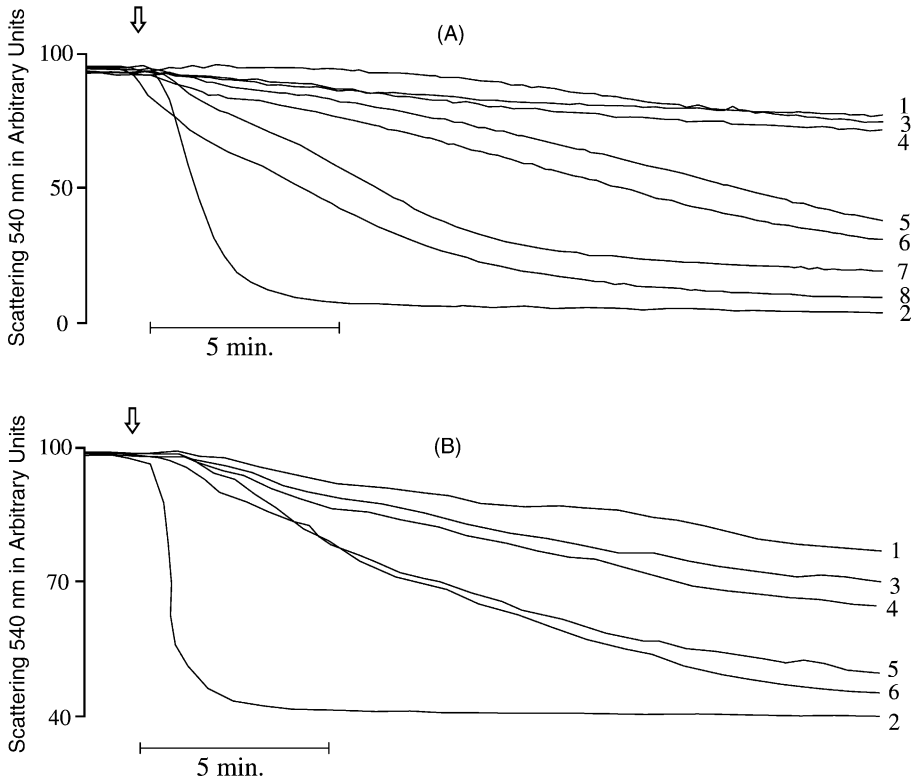

Fig. 5. The effect of CsA on amiodarone-induced mitochondrial swelling. Mitochondrial swelling was demonstrated by monitoring E_{540} in isolated rat liver (A) and heart (B) mitochondria. Swelling was induced at arrow by adding amiodarone at the indicated concentration together with or without CsA. (A) Line 1: baseline swelling (no agent); line 2: 60 μM Ca^{2+}-induced swelling; line 3: 10 μM Ad; line 4: 10 μM Ad + CsA; line 5: 20 μM Ad; line 6: 20 μM Ad + CsA; line 7: 30 μM Ad; line 8: 30 μM Ad + CsA. (B) Line 1: baseline swelling (no agent); line 2; 60 μM Ca^{2+}-induced swelling; line 3: 10 μM Ad; line 4: 10 μM Ad + CsA; line 5: 20 μM Ad; line 6: 20 μM Ad + CsA.

reports found no such effect [31]. To assess this issue, we monitored the real time *in situ* concentrations of creatine phosphate, ATP and inorganic phosphate during ischemia-reperfusion of Langendorff-perfused rat hearts by ^{31}P NMR spectroscopy. We have found that a single i.v. shot of 20 mg/kg amiodarone administered 30 min before the removal of the heart resulted in a faster and more complete recovery of the high-energy phosphate concentrations. According to previous studies [11,15], this administration protocol resulted in a rather low blood concentration of amiodarone, and the drug was predominantly in membrane bound form. Under these conditions, amiodarone had a significant protecting effect on the energy metabolism of perfused hearts during ischemia-reperfusion. This protecting effect was one of the most pronounced among more than 30 compounds, including Ca^{2+}-channel blockers, β blockers, free-radical scavengers, and inhibitors of the poly-(ADP-ribose) polymerase enzyme tested in this laboratory up to this time ([32,33], Toth, personal communication).

To assess the direct effect of amiodarone on cardiomyocytes, we performed viability studies on H9C2 rat cardiomyocyte cell line, in comparison to BRL-3A hepatocyte and PANC-1 pancreatic carcinoma cell lines. We chose cell lines with doubling time similar to the cardiomyocyte line, and representing extracardiac tissues of increased vulnerability toward amiodarone. (Toxicity of amiodarone on thyroid cells was assessed by another group [34], and non-fibroblast lung as well as normal pancreatic cell lines are unavailable.) Extracardiac cell lines had an enhanced sensitivity toward amiodarone toxicity in the whole concentration range tested (Fig. 2A). The toxicity of amiodarone was much higher on BRL-3A hepatocyte line than on PANC-1 cells; however, PANC-1 line is a carcinoma line, so certainly represent a less mature differentiation state than the normal hepatocyte line. The concentration range of 1–100 μM is comparable to those observed in different tissues during prolonged amiodarone treatment. Different concentrations of amiodarone was reported to induce apoptosis and cell death in different tissues and cell lines [6,34]. As we demonstrated by DNA laddering in SP-2 myeloma (Fig. 2B), the cell death induced by amiodarone was apoptotic. However, amiodarone did not present sign of apoptosis in H9C2 cells whereas it showed DNA fragmentation in the extracardiac cell line SP-2 in the same concentration range. The results of the viability study are further supported by the results gained from the oxygen consumption of the cell lines measured by a Clark electrode (Fig. 3B–D), showing that in extracardiac cell lines amiodarone inhibits the respiration in a lower concentration than in the case of H9C2 cells.

The molecular mechanism of the antiarrhythmic effect of amiodarone is well characterized [1,3]. However, its

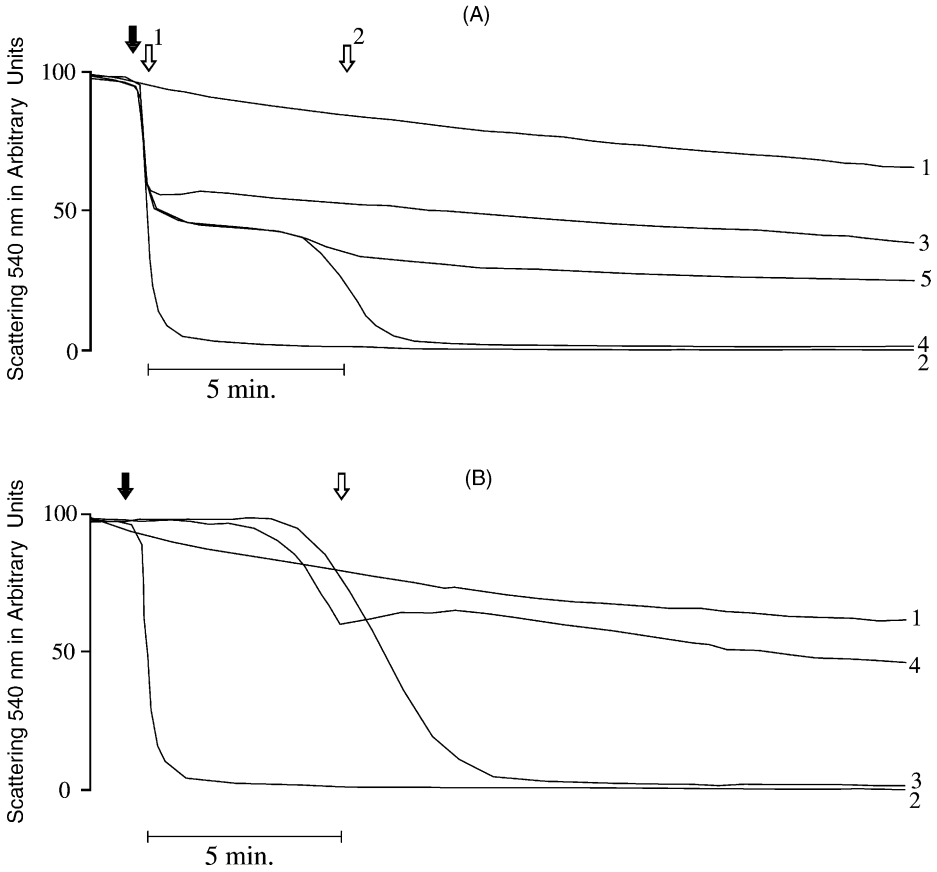

Fig. 6. The combined effect of amiodarone and CsA on Ca^{2+}-induced mitochondrial swelling. Swelling of isolated rat liver mitochondria was demonstrated by monitoring E_{540} and was induced at filled arrow by adding 60 μM Ca^{2+}. When indicated, Ad and CsA were added at first or second open arrow. (A) Line 1: baseline swelling (no Ca^{2+} added); line 2: no Ad or CsA; line 3: 2.5 μM CsA added at first open arrow; line 4: 0 μM Ad added at first open arrow; line 5: 10 μM Ad added at first open arrow then 2.5 μM CsA at second open arrow. (B) Line 1: baseline swelling (no Ca^{2+} added); line 2: no Ad or CsA; line 3: 10 μM Ad was present throughout the experiment; line 4: 2.5 μM CsA added at open arrow while 10 μM Ad was present throughout the experiment.

cardioprotectivity and cytotoxicity is poorly understood. Amiodarone was reported to inhibit anion and cation currents [35–37], affect membrane fluidity [38,39] and water permeability [40] and inhibit various membrane-associated enzymes and transporters such as Na^+/K^+ ATPase [39], mitochondrial ATPase [14], complexes I and II [41], phospholipase A1 [42], carnitine palmitoyl tranferase 1 [16], etc. These diverse effects of amiodarone can contribute to its toxic property.

The observation that amiodarone protected energy metabolism suggested that cardioprotectivity of the drug can be the consequence of its direct mitochondrial effects, such as its effect on mitochondrial respiration, reactive oxygen species production and permeability transition. Some reports addressed the effect of amiodarone on mitochondrial respiration and ROS production; however, the effect of the drug on permeability transition have not yet been studied, although involvement of latter in collapse of oxidative phosphorylation and ion homeostasis, as well as in mediation of both necrotic and apoptotic cell death is well established [17,18]. Therefore, we studied the effect of amiodarone on permeability transition using isolated, Percoll-gradient-purified liver and heart mitochondria.

We found that amiodarone exerted a concentration dependent effect on mitochondrial permeability transition. Up to the concentration of 10 μM, amiodarone inhibited the onset of Ca^{2+}-induced permeability transition, however, at higher concentrations, it induced a CsA-independent swelling. We demonstrated permeability transition by monitoring the accompanying mitochondrial swelling, and verified the opening of the permeability pore by an enzymatic method.

Mitochondrial swelling is usually monitored for about 5–10 min [27,43], so during a usual swelling experiment, the effect of amiodarone would have seemed identical to that of CsA. We have monitored mitochondrial swelling for more than 20 min and indeed observed a difference between the inhibitory effect of amiodarone and CsA. CsA was reported to prevent the onset of Ca^{2+}-induced swelling for at least 40 min while according to our results, up to 10 μM amiodarone delayed it for about 10 min indicating a different mechanism and probably a different site of action for amiodarone and CsA in inhibiting mitochondrial permeability transition. In contrast to the Ca^{2+}-induced one, the amiodarone-induced mitochondrial swelling was not inhibited by CsA, a specific inhibitor of the

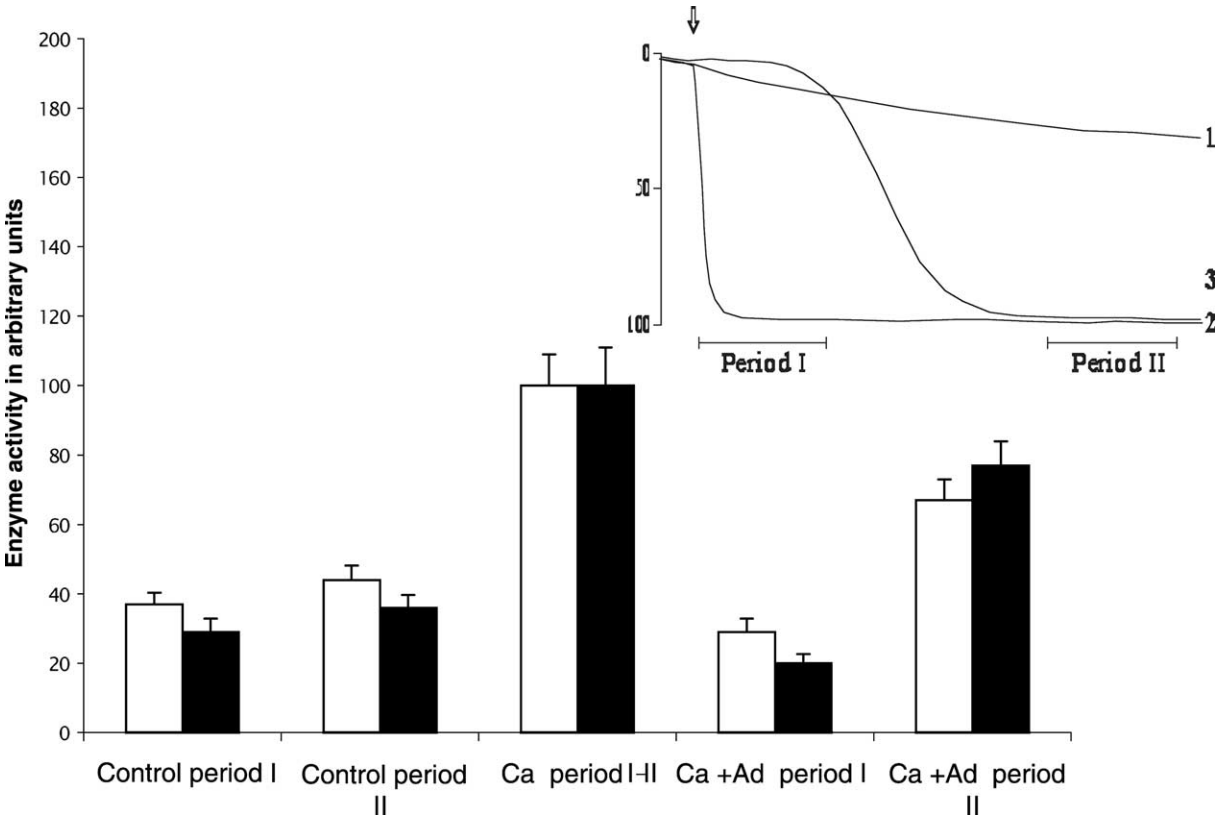

Fig. 7. Demonstration of the opening of the PTP in heart mitochondria. Opening of the PTP was demonstrated by measuring the activities of citrate synthase (filled bars) and carnitine acetyl transferase (open bars) enzymes in mitochondria exposed to no agent (insert line 1), 60 μM Ca^{2+} (insert line 2), or 60 μM Ca^{2+} in the presence of 10 μM Ad (insert line 3) during period I and II of a Ca^{2+}-induced swelling experiment. On an insert at the upper right corner of the figure the appropriate swelling curves are presented in order to indicate the timing of the enzyme activity measurements. Data represents average ± SEM of at least five independent experiments.

adenine nucleotide translocase-cyclophilline D complex of the mitochondrial permeability pore. The inhibitory effect of up to 10 μM amiodarone and the inhibitory effect of CsA on the Ca^{2+}-induced permeability transition seemed additive (Fig. 5). These results suggests that amiodarone exerted its effect on a site distinct from the known PTP. Amiodarone had inhibitory effect on complex I [12]; therefore, we analyzed the effect of rotenone, and found that it inhibits the Ca^{2+}-induced swelling in low concentration, but unlike amiodarone it does not induce swelling on its own in higher concentrations. Amiodarone also had uncoupling effect, so we analyzed the effect of low concentrations of FCCP on the swelling. FCCP, in low concentrations, completely inhibited the Ca^{2+}-induced mitochondrial swelling, while it did not induce swelling on its own. This indicates that though amiodarone has both uncoupling and inhibitory effect, it's effect on mitochondrial swelling is exerted probably by a different mechanism.

The effect of amiodarone on states 3 and 4 respiration with succinate as substrate was also dependent of its concentration. In isolated mitochondria, at low concentrations of up to 6 μM, it did not affect, in the intermediate concentration range of 6–30 μM, it uncoupled, while at higher concentrations, it inhibited the oxygen consumption as measured by a Clark electrode (Fig. 3A). It is noteworthy that at the concentrations where amiodarone did not affect the respiration, it inhibited the Ca^{2+}-induced permeability transition ($IC_{50} = 3.9 \pm 0.8$ μM). We checked whether opening of the permeability pore could have been the cause of the uncoupling and inhibitory effect of amiodarone. To this end, we measured respiration in the presence of Ca^{2+} concentrations that were more than enough to induce permeability transition. Although the conditions did not differ considerably for the measurements of respiration and permeability transition (we omitted EGTA from the Clark buffer when studied the effect of Ca^{2+}), we could not detect any effect of up to 300 μM Ca^{2+} on the respiration (data not shown). It indicates, that under the conditions we used, the induction of permeability transition could not cause the uncoupling and inhibition of the respiration.

Since agents affecting the oxidative phosphorylation can induce ROS production that in turn can lead to mitochondrial permeability transition [44], we measured amiodarone-induced ROS formation in both the aqueous and the lipid phase of isolated mitochondria. In accordance to previous results [12,13,34], we could not detect ROS formation induced by up to 100 μM amiodarone indicating that ROS did not participate in mediating the effects of amiodarone.

Here, we present evidence that amiodarone has beneficial effects on the level of high-energy phosphates of the heart during ischemia and reperfusion as detected by real time *in situ* ^{31}P NMR measurements. We demonstrated a difference in sensitivity of cardiomyocytes and extracardiac cell lines toward the drug. We also report for first time that amiodarone affects mitochondrial permeability transition in a concentration dependent way. When present at low concentration where amiodarone does not have either uncoupling or inhibitory effect on respiration, it can protect the energy metabolism of post-ischemic heart by inhibiting the mitochondrial swelling induced by the elevated intracellular Ca^{2+}. On the other hand, at higher concentrations, besides uncoupling or inhibiting mitochondrial respiration, by a different mechanism, it can also damage tissues by inducing mitochondrial swelling leading to apoptotic cell death. Thus, the concentration dependent nature of amiodarone's effect on mitochondrial swelling together with the different sensitivities of the tissues toward amiodarone could be involved in the beneficial cardiac and the simultaneous toxic extracardiac effects of the drug.

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