

Protective Effect of Amiodarone but Not *N*-Desethylamiodarone on Postischemic Hearts through the Inhibition of Mitochondrial Permeability Transition

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ABSTRACT

Amiodarone is a widely used and potent antiarrhythmic agent that is metabolized to desethylamiodarone. Both amiodarone and its metabolite possess antiarrhythmic effect, and both compounds can contribute to toxic side effects. Here, we compare the effect of amiodarone and desethylamiodarone on mitochondrial energy metabolism, membrane potential, and permeability transition and on mitochondria-related apoptotic events. Amiodarone but not desethylamiodarone protects the mitochondrial energy metabolism of the perfused heart during ischemia in perfused hearts. At low concentrations, amiodarone stimulated state 4 respiration due to an uncoupling effect, inhibited the Ca^{2+} -induced mitochondrial swelling, whereas it dissipated the mitochondrial membrane potential ($\Delta\Psi$), and prevented the ischemia-reperfusion-induced release of apoptosis-inducing factor (AIF). At higher concentrations, amiodarone inhibited the mitochondrial respiration and simulated a

cyclosporin A (CsA)-independent mitochondrial swelling. In contrast to these, desethylamiodarone did not stimulate state 4 respiration, did not inhibit the Ca^{2+} -induced mitochondrial permeability transition, did not induce the collapse of $\Delta\Psi$ in low concentrations, and did not prevent the nuclear translocation of AIF in perfused rat hearts, but it induced a CsA-independent mitochondrial swelling at higher concentration, like amiodarone. That is, desethylamiodarone lacks the protective effect of amiodarone seen at low concentrations, such as the inhibition of calcium-induced mitochondrial permeability transition and inhibition of the nuclear translocation of the proapoptotic AIF. On the other hand, both amiodarone and desethylamiodarone at higher concentration induced a CsA-independent mitochondrial swelling, resulting in apoptotic death that explains their extracardiac toxic effect.

Amiodarone (2-butyl-3-benzofuranyl 4-[2-(diethylamino)-ethoxy]-3,5-diiodophenyl-ketone hydrochloride) is one of the most effective antiarrhythmic drugs and is frequently used in the clinical practice for treating ventricular and supraventricular arrhythmias. It is a class III antiarrhythmic agent, prolonging action potential duration whose effect may involve blocking of β -adrenergic receptors, sodium channels, and L-type calcium channels (Singh and Vaughan Williams, 1970; Nokin et al., 1983; Nattel et al., 1987; Varro et al., 1996). It may also have a role in preventing mortality after myocardial infarction (Julian et al., 1997). Despite its effec-

tive antiarrhythmical properties, the use of amiodarone is often limited by its toxic side effects, including thyroid dysfunction, liver, and pancreas fibrosis (Amico et al., 1984; Martin and Howard, 1985). However, the most severe adverse effect of the drug is pulmonary fibrosis, occurring in up to 13% of the patients receiving the amiodarone in doses higher than 400 mg day^{-1} (Martin and Rosenow, 1988). The etiology of the amiodarone-induced pulmonary toxicity is unknown.

Desethylamiodarone, the major metabolite of amiodarone, also has antiarrhythmic activity, significantly increasing the action potential duration (class III antiarrhythmic effect) and decreasing the maximum rate of depolarization (class I antiarrhythmic effect) at clinically relevant concentrations (Palandi and Campbell, 1987). This antiarrhythmic effect was shown to be in part dependent on gene expression rather than a direct effect on cell membrane channels or receptors

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ABBREVIATIONS. $\Delta\Psi$, mitochondrial membrane potential; AIF, apoptosis-inducing factor; ROS, reactive oxygen species; CsA, cyclosporin A; Rh123, rhodamine 123; DRh123, dihydrorhodamine 123; Dea, desethylamiodarone; MTT^+ , 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; ANOVA, analysis of variance; FCCP, carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone.

(Drvota et al., 1998). Desethylamiodarone rapidly accumulates in the lung after amiodarone treatment, sometimes in higher concentrations than amiodarone itself (Daniels et al., 1989). It proved to be more toxic than amiodarone in pulmonary cell types (Ogle and Reasor, 1990), suggesting that desethylamiodarone may play an important role in the development of the amiodarone treatment-induced pulmonary fibrosis.

Whereas numerous studies support the toxic effect of amiodarone and its metabolite desethylamiodarone on extracardiac tissues such as lung, thyroid gland, liver, and pancreas, reports on the effects of amiodarone on cardiac function have been variable. Some emphasized its beneficial effect on cardiac functions and arrhythmia after ischemia and reperfusion (Nokin et al., 1987; Vander-Elst et al., 1990); however, other reports found it worsened the damage to the mitochondrial energy metabolism caused by ischemia-reperfusion (Moreau et al., 1999). Moreover, there are only limited data about the effect of desethylamiodarone on ischemic heart. We have previously demonstrated that amiodarone increases the level of the high-energy phosphate metabolites by directly influencing the mitochondria after ischemia and reperfusion in perfused rat hearts (Varbiro et al., 2003). Myocardial ischemia can lead to a severe arrhythmia that may necessitate amiodarone administration; therefore, it is important to assess the effect of amiodarone and its metabolite desethylamiodarone on postischemic heart. Because ischemia most of all deteriorates the energy metabolism of the mitochondria, it is especially important to evaluate their effect on the mitochondrial functions. In addition to their critical role in energy metabolism, mitochondria are known to regulate cell viability as well as cell death (Kroemer and Reed, 2000) through pathways such as mitochondrial permeability transition with the dissipation of the mitochondrial membrane potential ($\Delta\Psi$) and release of proapoptotic factors such as cytochrome *c* or the apoptosis-inducing factor (AIF), the disruption of ATP production, and the generation of reactive oxygen species (ROS).

In this study, we aimed to verify that in low concentrations amiodarone but not desethylamiodarone has cardioprotective features, whereas at higher concentrations they both exhibit toxic properties especially to extracardiac tissues. Therefore, we examined the effect of amiodarone and desethylamiodarone on mitochondrial energy metabolism during ischemia-reperfusion of Langendorff-perfused rat hearts by *in situ* ^{31}P NMR spectroscopy. We assessed and compared the cytotoxicity of amiodarone and desethylamiodarone on cardiomyocytes as well as on extracardiac cells in culture. In an attempt to reveal the underlying mechanism of amiodarone and desethylamiodarone in these paradigms, we studied their effect on the translocation of the proapoptotic AIF from the mitochondria to the nucleus of the perfused heart cells; their direct mitochondrial effects, including the induction of ROS production; the effect on the $\Delta\Psi$ and the opening of mitochondrial permeability transition pore; and their effect on the mitochondrial respiration.

Materials and Methods

Chemicals. Cyclosporin A (CsA) was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Rhodamine 123 (Rh123) and dihydrorhodamine123 (DRh123) were from Molecular Probes (Eu-

gene, OR), and desethylamiodarone (Dea) was a gift from Professor Varro (Department of Pharmacology and Pharmacotherapy, University of Szeged, Szeged, Hungary). All other compounds were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Animals. Wistar rats were purchased from Charles River Hungary Breeding Ltd. (Budapest, Hungary). 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.

Cell Culture. PANC-1 human pancreatic epithelioid carcinoma cells, BRL 3A rat liver cells, H9C2 mouse cardiomyocytes, and WRL-68 human liver cells were from American Type Culture Collection (Manassas, VA). The cell lines were grown in humidified 5% CO_2 atmosphere at 37°C. The cells were maintained as monolayer adherent culture in Dulbecco's modified Eagle's medium containing 1% antibiotic-antimycotic solution (Sigma Chemical, Poole, Dorset, UK) and 10% fetal calf serum. Cells were passaged at intervals of 3 days.

Heart Perfusion. Pretreatment with amiodarone or desethylamiodarone was done exactly as described previously (Nokin et al., 1987). Briefly, adult male Wistar rats (weighing 300–350 g; $n = 4$ in each group) were anesthetized with 200 mg/kg ketamine *i.p.* and then either 400 $\mu\text{l}/\text{kg}$ isotonic saline (sham-operated), 20 mg/kg amiodarone (400 $\mu\text{l}/\text{kg}$ Cordarone; Sanofi-Synthelabo Budapest, Hungary), or 20 mg/kg desethylamiodarone was injected into the femoral vein. Thirty minutes after the treatment, the animals were heparinized with sodium heparin (100 IU/rat *i.p.*), and their hearts were excised and merged into ice-cold Krebs-Henseleit buffer. Hearts were perfused via the aorta according to the Langendorff method at a constant pressure of 70 mm Hg, at 37°C as described previously (Szabados et al., 1999). The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer consisting of 118 mM NaCl, 5 mM KCl, 1.25 mM CaCl_2 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 , 11 mM glucose, and 0.6 mM octanoic acid. The perfusate was adjusted to pH 7.4 and bubbled with 95% O_2 , 5% CO_2 through a glass oxygenator. After a washout (nonrecirculating 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 spectroscopy during the entire perfusion.

Determination of Drug Concentrations. The concentrations of amiodarone and *N*-desethylamiodarone in the plasma and in the heart mitochondria of rats 30 min after the pretreatment by either 20 mg/kg amiodarone or desethylamiodarone ($n = 3$ in each group) were determined by a high-performance liquid chromatographic procedure as described previously (Kannan et al., 1987).

NMR Spectroscopy. NMR spectra were recorded with a Varian UNITY INOVA 400 WB instrument. ^{31}P measurements (161.90 MHz) of perfused hearts were run at 37°C in a Z-SPEC 20-mm broadband probe (Nalorac Co., Martinez, CA), applying WALTZ proton decoupling ($\gamma\text{B}_2 = 1.6$ kHz) during the acquisition only. Field homogeneity was adjusted by following the ^1H signal ($w_{1/2} = 10$ –15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each free-induction decay. Flip angle pulses (45°) were used after a 1.25-s recycle delay, and transients were acquired over a 10-kHz spectral width in 0.25 s, and the acquired data points (5000) were zero-filled to 16384.

Under the abovementioned circumstances, relative concentrations of the species are proportional to the corresponding peak areas, because interpulse delays exceeded by 4 to 5 times the T_1 values of the metabolites that were analyzed in the ^{31}P experiments. Data were acquired from five independent experiments for sham-operated and amiodarone-treated groups each.

Western Blot Analysis of AIF. Myocardial specimens ($n = 3$ in each group) were snap-frozen immediately after surgical removal or at the end of the Langendorff perfusion experiment and stored at -80°C until analyzed. Frozen heart muscle samples were mechanically homogenized in liquid nitrogen and the nuclear fraction was

prepared as described previously (Schmitt et al., 2002). Equal amounts of nuclear extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gel) and then transferred to a nitrocellulose filter. Membranes were blocked using 5% dry milk. The blot was probed with monoclonal antibodies against AIF (Oncogene Science, Cambridge, MA) overnight at the temperature of 4°C. The antigen-antibody complex was visualized on an X-ray film using secondary antibodies linked to horseradish peroxidase (1:1000; Sigma-Aldrich) and a chemiluminescence kit (ECL; Amersham Biosciences Inc., Piscataway, NJ). The experiments were repeated three times for each group, and the results are demonstrated by photomicrograph of a representative blot.

Cell Viability Assay. PANC 1, BRL-3A, WRL-68, and H9C2 cells were seeded into 96-well plates at a starting density of 2.5×10^4 cell/well and cultured overnight in humidified 5% CO₂ atmosphere at 37°C. The next day, amiodarone or desethylamiodarone at the indicated concentrations were added to the medium. Forty-eight hours later, 0.5% of the water-soluble mitochondrial dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT⁺) was added. Incubation was continued for three more hours, the medium was removed, and the water-insoluble blue formazan dye formed stoichiometrically from MTT⁺ was solubilized by acidic isopropanol. Optical densities were determined by a 2010 ELISA reader (Anthos Labtech, Vienna, Austria) at 550-nm wavelength. All experiments were run in at least four parallels and repeated three times.

Isolation of Mitochondria. Liver and heart mitochondria were prepared according to standard protocol (Schneider and Hageboom, 1950). The only difference among the organs was in the primary homogenization protocol; liver was squeezed through a liver press, whereas pooled heart tissue from five rats was minced with a blender. All isolated mitochondria were purified by Percoll gradient centrifuging (Sims, 1990).

Mitochondrial oxygen consumption was detected by a Clark electrode. Briefly, isolated mitochondria were suspended in 20 mM Tris buffer, pH 7.4, containing 20 mM KCl, 220 mM mannitol, 70 mM sucrose, and 1 mM EGTA. The mitochondrial respiration (state 4) was assessed with either 10 mM pyruvate (complex I respiration) or 10 mM succinate (in the presence of 1 μM rotenone; complex II respiration) and different concentrations of amiodarone or desethylamiodarone. Before performing experiments, respiratory control ratio 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 (Elimadi et al., 1997; Garcia et al., 1997). The results are expressed as mean values \pm S.E.M. from three independent experiments.

Mitochondrial permeability transition was monitored by after the accompanying large-amplitude swelling via the decrease in absorbance at 540 nm (Cassarino et al., 1999) measured at room temperature by a fluorimeter (PerkinElmer Life Sciences, London, UK) in reflectance mode. Briefly, mitochondria at the concentration of 1 mg protein/ml were preincubated in the assay buffer (70 mM sucrose, 214 mM mannitol, 20 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid, 5 mM glutamate, 0.5 mM malate, and 0.5 mM phosphate) containing the studied substances for 60 s. Mitochondrial permeability transition was induced by the addition of either 60 μM Ca²⁺, or amiodarone or desethylamiodarone at the indicated concentration. Decrease of E₅₄₀ was 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.

Mitochondrial membrane potential was monitored by fluorescence of Rh123, released from the mitochondria after the induction of permeability transition at room temperature by using a fluorimeter (PerkinElmer Life Sciences) at an excitation wavelength of 495 nm

and an emission wavelength of 535 nm. Briefly, mitochondria at the concentration of 1 mg of protein/ml were preincubated in the assay buffer (70 mM sucrose, 214 mM mannitol, 20 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid, 5 mM glutamate, 0.5 mM malate, and 0.5 mM phosphate) containing 1 μM Rh123 and the studied substances for 60 s. Alteration of ΔΨ was induced by the addition of either 60 μM of Ca²⁺, or amiodarone or desethylamiodarone at the indicated concentration. Changes of fluorescence intensity were detected for 4 min. The results are demonstrated by representative original registration curves from five independent experiments, each repeated three times using mitochondria prepared from the same liver or pool of rat hearts, respectively.

The Determination of ROS Formation. ROS formation was detected as described previously (Varbiro et al., 2001) by the fluorescence of Rh123 formed by ROS-induced oxidation of the nonfluorescent DRh123 in situ at an excitation wavelength of 495 nm and an emission wavelength of 535 nm by a fluorimeter (PerkinElmer Life Sciences). 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.

Statistical Analysis. Data were presented as means \pm S.E.M. For multiple comparisons of groups, ANOVA was used. Statistical difference between groups was established by paired or unpaired Student's *t* test, with Bonferroni correction.

Results

Effect of Amiodarone and Desethylamiodarone 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 ³¹P NMR spectroscopy. To study the effect of amiodarone or desethylamiodarone on the energy metabolism of the perfused hearts, a single i.v. injection of 20 mg/kg amiodarone or 20 mg/kg desethylamiodarone 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. This resulted in a mitochondrial amiodarone and desethylamiodarone concentrations of 2.42 ± 0.29 and 2.69 ± 0.28 μg/mg mitochondrial protein, respectively (Table 1). 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, whereas creatine phosphate concentrations in hearts of sham-operated animals recovered to $35 \pm 4\%$ of their normoxic level. Amiodarone pretreatment resulted in a significantly higher

TABLE 1

Concentration values for amiodarone and desethylamiodarone in the plasma and the heart mitochondria of rats measured by high-performance liquid chromatography 30 min after the i.v. administration of either 20 mg/kg amiodarone or desethylamiodarone. Results are expressed as mean \pm S.E.M. (*n* = 3).

	Plasma	Heart Mitochondria
	μg/ml \pm S.E.M.	μg/mg \pm S.E.M.
Amiodarone	4.1 ± 0.8	2.42 ± 0.29
Desethylamiodarone	4.3 ± 4.2	2.69 ± 0.28

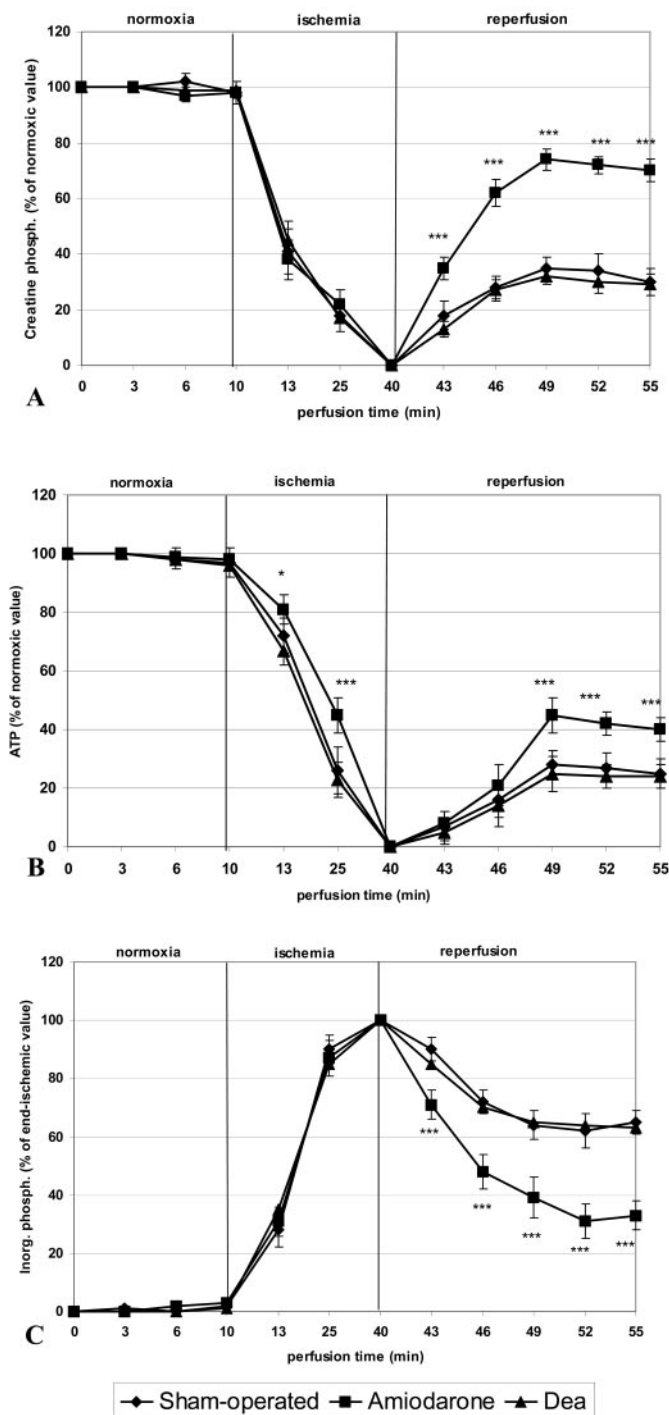


Fig. 1. Effect of amiodarone and desethylamiodarone on the high-energy phosphate metabolism in Langendorff-perfused rat hearts. Groups of five rats were treated by a single shot of physiological salt solution (sham-operated), 20 mg/kg amiodarone, or 20 mg/kg desethylamiodarone i.v. 30 min before sacrifice. Their hearts were removed and applied to a Langendorff perfusion apparatus, which was inserted into the magnet of a 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 represent average \pm S.E.M. Note that the time axis is not proportional. *, $p < 0.05$ (mean \pm S.E.M., repeated measures ANOVA); ***, $p < 0.001$ (mean \pm S.E.M., repeated measures ANOVA) of amiodarone from equimolar concentrations of desethylamiodarone.

recovery ($p < 0.001$) of creatine phosphate concentrations during the ischemia-reperfusion cycle ($74 \pm 6\%$; Fig. 1A)

compared with control ($35 \pm 4\%$; Fig. 1A), whereas the pretreatment with desethylamiodarone had no significant effect on the recovery of creatine phosphate concentration after ischemia ($32 \pm 4\%$; Fig. 1A). In addition, amiodarone pretreatment slightly but significantly ($p < 0.05$) delayed the decrease of ATP concentrations during ischemia, whereas it significantly ($p < 0.001$) improved the recovery of ATP level ($45 \pm 6\%$ versus $28 \pm 5\%$; Fig. 1B). The pretreatment with desethylamiodarone had no significant effect on the recovery of ATP level ($28 \pm 5\%$; Fig. 1B) compared with control. Amiodarone pretreatment facilitated the significantly ($p < 0.001$) faster and more complete utilization of inorganic phosphate ($31 \pm 6\%$ versus $62 \pm 6\%$; Fig. 1C) during reperfusion. The pretreatment with desethylamiodarone did not prove to be effective in the utilization of inorganic phosphate ($63 \pm 4\%$; Fig. 1C).

Effect of Amiodarone and Desethylamiodarone on AIF Translocation in Perfused Hearts after Ischemia-Reperfusion. The release and nuclear translocation of AIF was detected from the nucleus of Langendorff-perfused rat heart tissue after ischemia and reperfusion by Western blot. The results, demonstrated by a photomicrograph of a representative blot, are presented in Fig. 2. The removal and normoxic perfusion of the heart increases the level of AIF in the nucleus of the heart cell compared with the control (Fig. 2, lanes 1 and 2); however, this is further increased by 30 min of ischemia and 60 min of reperfusion (Fig. 2, lane 3). Pretreatment with a single i.v. injection of 20 mg/kg amiodarone prevented the increase in the level of AIF stimulated by ischemia-reperfusion (Fig. 2, lane 4). The pretreatment with a single i.v. injection of 20 mg/kg desethylamiodarone, however, does not have any attenuative effect on the increase of AIF level after ischemia-reperfusion (Fig. 2, lane 5).

Effect of Amiodarone and Desethylamiodarone on Cultured Cell Lines. Viability of H9C2, BRL-3A, WRL-68, and PANC-1 cells exposed to different concentrations of amiodarone or desethylamiodarone for 48 h were assessed by the MTT^+ method. In each of the cell lines desethylamiodarone proved to be more toxic than amiodarone in a specific concentration range (Fig. 3). This concentration range seemed to be variable in the different cell lines. The significant differ-

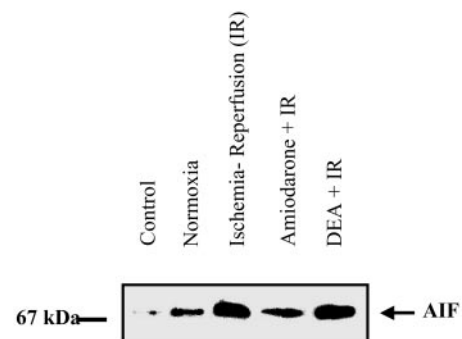


Fig. 2. Effect of amiodarone and desethylamiodarone on the nuclear AIF level in Langendorff-perfused rat hearts. Western blot analysis of AIF level in the nuclear fraction of rat heart tissue after ischemia-reperfusion in Langendorff-perfused rat hearts. Lane 1, control (no perfusion); lane 2, 30 min of normoxic perfusion; lane 3, 30 min of ischemia followed by 60 min of reperfusion; lane 4, pretreatment with 20 mg/kg amiodarone for 30 min followed by excision of the heart and 30 min of ischemia and 60 min of reperfusion; and lane 5, pretreatment with 20 mg/kg desethylamiodarone for 30 min followed by excision of the heart and 30 min of ischemia and 60 min of reperfusion.

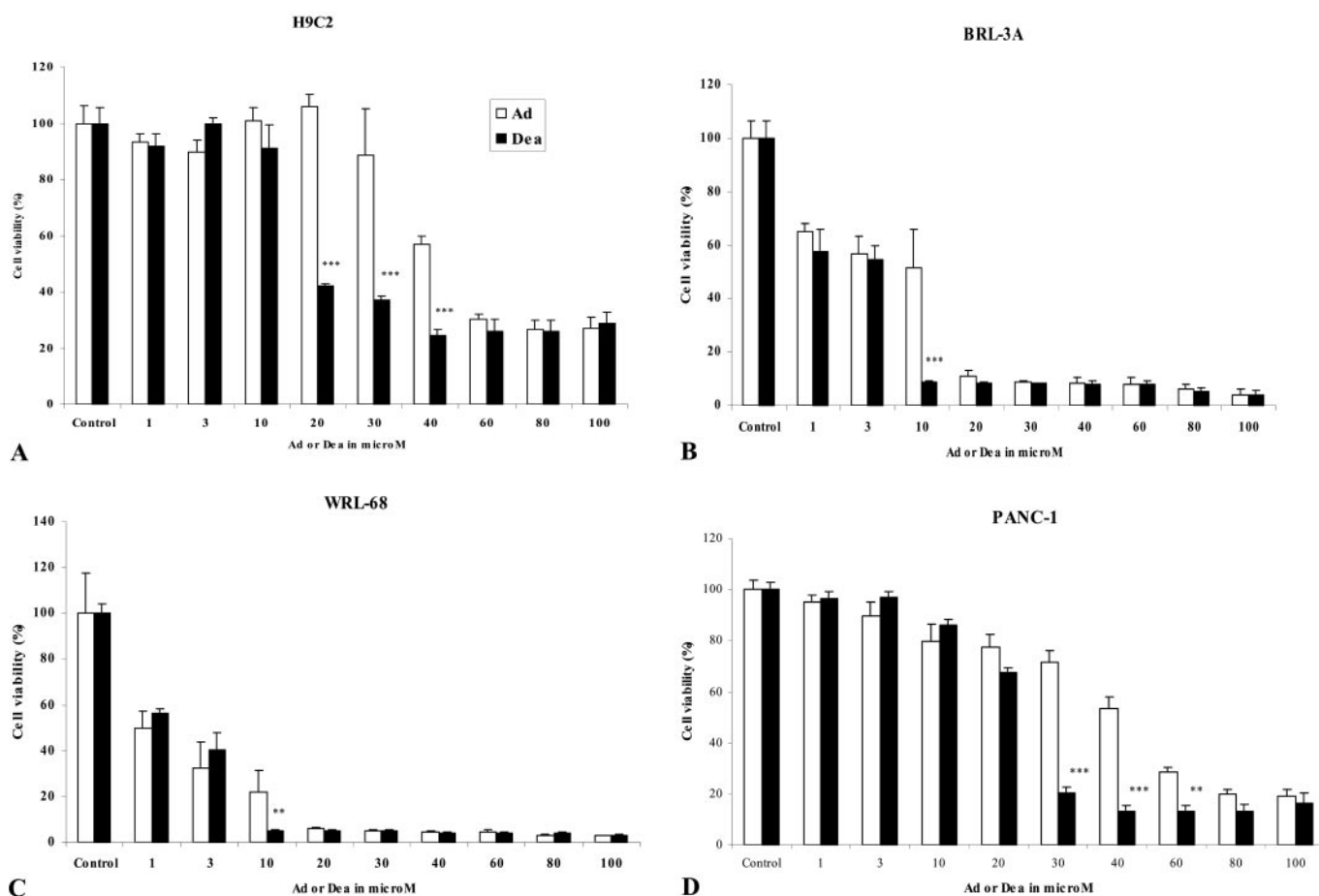


Fig. 3. Effect of amiodarone and desethylamiodarone on viability of cell lines. The effect of amiodarone (open columns) and desethylamiodarone (filled columns) on viability of H9C2 (A), BRL-3A (B), WRL-68 (C), and HeLa cells (D) were detected by the formation of water-insoluble blue formazan dye from the yellow mitochondrial dye MTT^+ by the functionally active mitochondria of the cells. The cells were exposed to different concentrations of amiodarone or desethylamiodarone for 48 h before the addition of the MTT^+ dye. Data represent average \pm S.E.M. of three independent experiments running in four parallels. **, significant difference ($p < 0.01$; mean \pm S.E.M., paired t test); ***, significant difference ($p < 0.001$; mean \pm S.E.M., paired t test) of amiodarone from equimolar concentrations of desethylamiodarone.

ence ($p < 0.001$) between the toxicity of amiodarone and desethylamiodarone observed in H9C2 cardiomyocytes was in a concentration range between 10 to 60 μM (Fig. 3A). In the normal rat (BRL-3A) and human (WRL-68) liver cell lines the significant difference ($p < 0.001$ and $p < 0.01$, respectively) between the toxicity of the drugs occurred in a lower concentration range between 3 to 20 μM (Fig. 3, A and B). In PANC-1 human pancreatic epithelioid carcinoma cells, significant difference ($p < 0.01$) between the toxicity of amiodarone and desethylamiodarone was observed in a concentration range of 20 to 80 μM as revealed by the viability data (Fig. 3D). The various cell lines presented different sensitivity toward the toxicity of amiodarone or desethylamiodarone (Table 2). The drugs were shown to be the least toxic in H9C2 cardiomyocyte cells. BRL-3A and WRL-68 hepatocytes were much more sensitive to amiodarone or desethylamiodarone toxicity than the PANC-1 human pancreatic epithelioid carcinoma cells; however, BRL-3A and WRL-68 were normal, whereas PANC-1 was a cancer cell line.

When H9C2 cardiomyocytes were exposed to different concentrations of amiodarone, desethylamiodarone, or FCCP for only 1 h, the results detected by the formation of water-insoluble blue formazan dye from the yellow mitochondrial dye MTT^+ by the functionally active mitochondria reflect the

TABLE 2

LD₅₀ values for amiodarone and desethylamiodarone in different cell lines after 48 h of incubation

Cell Lines	Amiodarone	Desethylamiodarone
	$\mu M \pm S.E.M.$	$\mu M \pm S.E.M.$
H9C2	49.1 \pm 5.8	17.2 \pm 3.9 ^a
BRL-3A	13.3 \pm 4.2	4.2 \pm 1.1 ^a
WRL-68	2.4 \pm 0.4	2.5 \pm 0.3
PANC-1	41.4 \pm 3.7	23.5 \pm 1.4 ^a

^a Significant differences between the LD₅₀ of amiodarone and desethylamiodarone ($p < 0.01$, paired t test, $n = 8$).

respiratory state, rather than the number of viable cells. Amiodarone, up to the concentration of 30 μM , exerted an uncoupling effect as indicated by the increase of optical densities, similar to that of the equimolar concentrations of FCCP, a widely used uncoupling agent (Fig. 4A). However, at the concentration of 100 μM , the stimulatory effect of amiodarone declined significantly ($p < 0.001$), compared with equimolar concentrations of FCCP (114 \pm 7 versus 141 \pm 6%; Fig. 4A). Desethylamiodarone did not exhibit any stimulatory effect on the respiration, when present in low concentrations, whereas it significantly ($p < 0.001$) decreased the MTT^+ formation, compared with equimolar amount of ami-

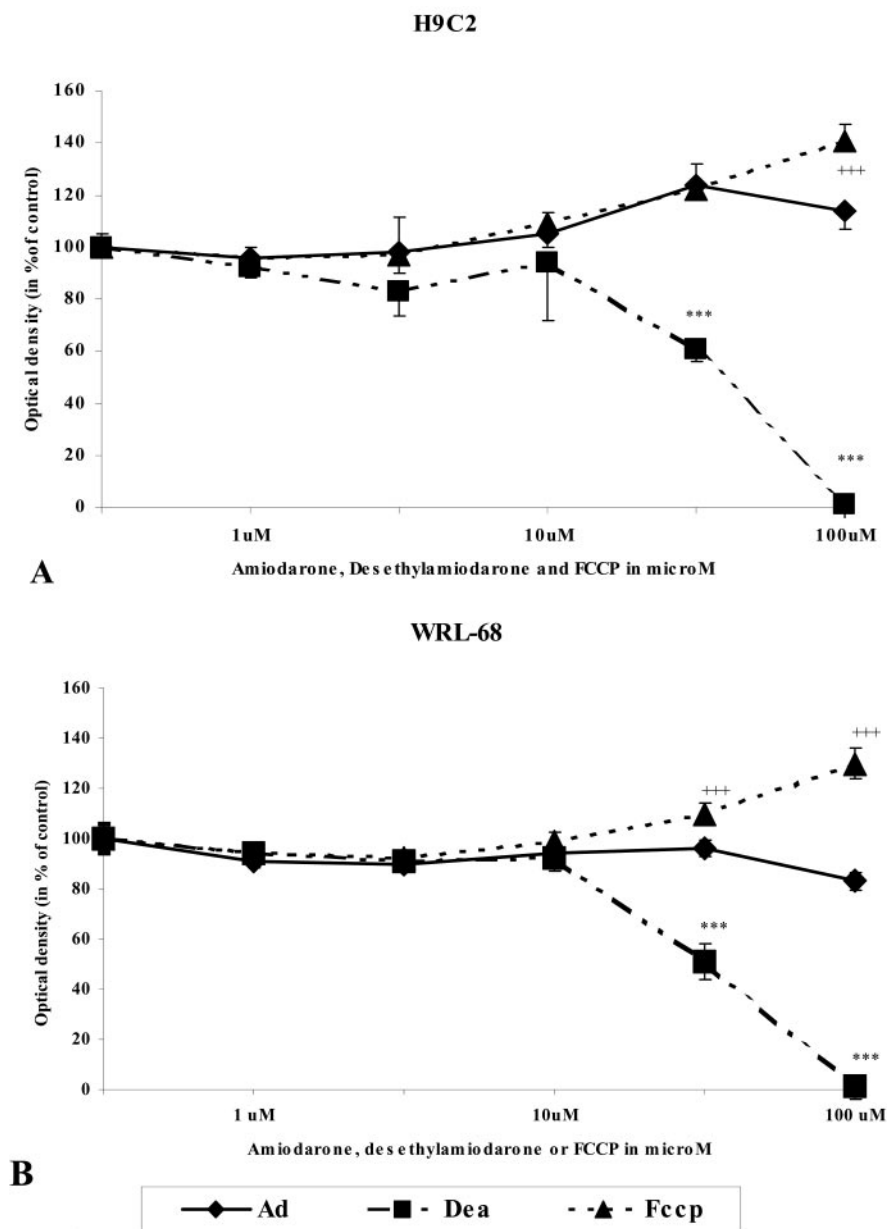


Fig. 4. Effect of amiodarone, desethylamiodarone, and FCCP on the cellular respiration. The effect of amiodarone, desethylamiodarone, and FCCP on respiration of H9C2 (A) and WRL-68 (B) cells was detected by the formation of water-insoluble blue formazan dye from the yellow mitochondrial dye MTT⁺ by the functionally active mitochondria of the cells. The cells were exposed to different concentrations of amiodarone, desethylamiodarone, or FCCP for 1 h before the addition of the MTT⁺ dye. Data represent average \pm S.E.M. of three independent experiments running in four parallels. +++, $p < 0.001$ (mean \pm S.E.M., ANOVA) of FCCP from equimolar concentrations of amiodarone; ***, $p < 0.001$ (mean \pm S.E.M., ANOVA) of desethylamiodarone from equimolar concentrations of amiodarone.

odarone above the concentration of 10 μM (Fig. 4A). In WRL-68 liver cells, amiodarone did not show any stimulatory effect up to the concentration of 30 μM , whereas it decreased the respiration, when present in higher concentration (Fig. 4B). The effect of both desethylamiodarone and FCCP on the respiration of WRL-68 was similar like in the case of H9C2, with desethylamiodarone showing inhibition but FCCP manifesting an uncoupling effect above the concentration of 10 μM (Fig. 4B). The values of the optical densities in this concentration range for both desethylamiodarone and FCCP were significantly different ($p < 0.001$) from those of the equimolar concentrations of amiodarone (Fig. 4B).

Effect of Amiodarone and Desethylamiodarone on the Mitochondrial Oxygen Consumption. The oxygen consumption of isolated mitochondria (state 4 respiration) was measured by a Clark electrode, with 10 mM pyruvate (complex I-supported respiration) or 10 mM succinate in the presence of 1 μM rotenone (complex II-supported respiration)

exposed to different concentrations of amiodarone or desethylamiodarone. In isolated rat heart mitochondria, at low concentrations of up to 6 μM in the case of complex I- and complex II-supported respiration, amiodarone did not have any significant effect, compared with the control. In an intermediate concentration range of 6 to 30 μM , amiodarone increased both the complex I- and complex II-supported oxygen consumption, indicating an uncoupling effect, whereas the drug gradually inhibited respiration at higher concentrations (Fig. 5, A and B).

Desethylamiodarone did not have any significant effect on the mitochondrial oxygen consumption at low concentrations of up to 10 μM . Above this concentration, it gradually inhibited the respiration supported by succinate and in the concentration above 30 μM the respiration supported by pyruvate, without presenting an uncoupling effect, as indicated by the absence of the stimulation of both complex I- and complex II-supported respiration in the concentration range

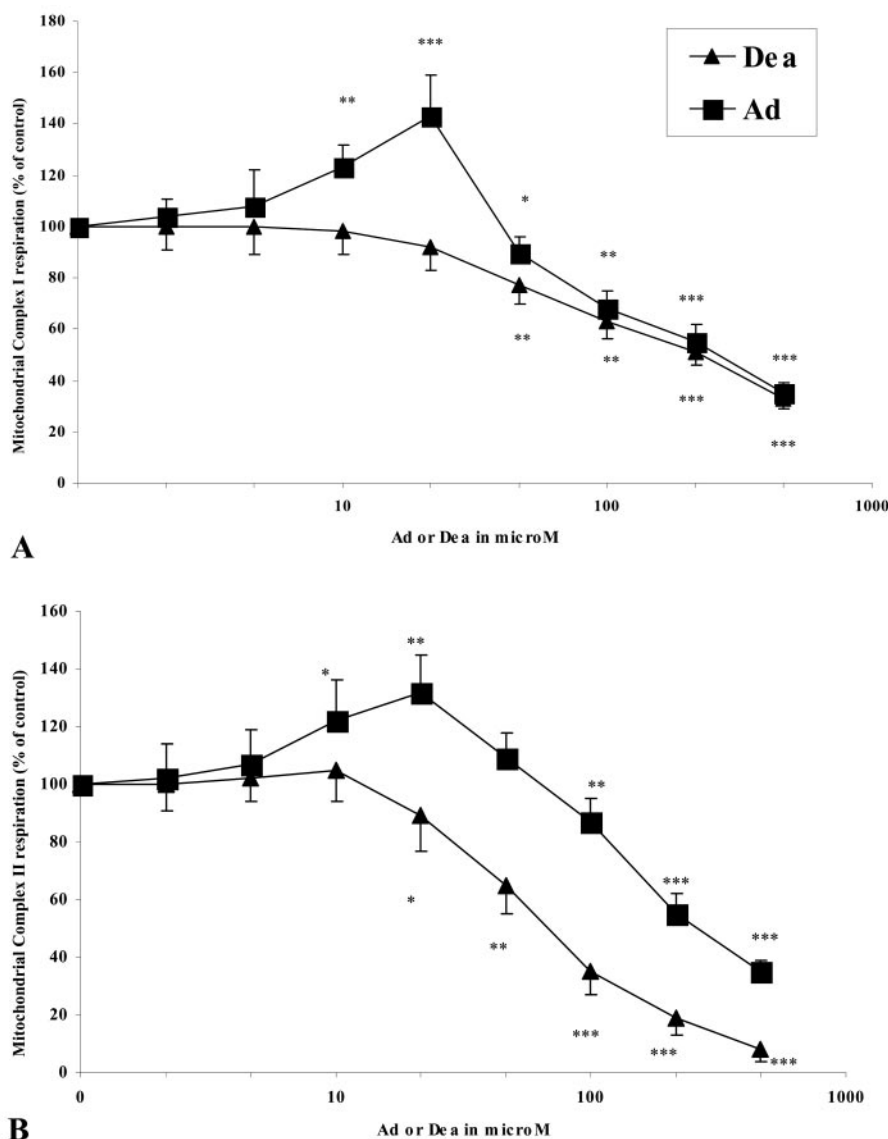


Fig. 5. Effect of amiodarone and desethylamiodarone on mitochondrial oxygen consumption. Mitochondrial respiration was measured by a Clark electrode and was supported with either 10 mM pyruvate (complex I respiration) or 10 mM succinate (complex II respiration) in the presence of different concentrations of amiodarone or desethylamiodarone. Data represent average \pm S.E.M. of three independent experiments repeated twice using mitochondria prepared from the same heart samples. Note that the concentration axis is logarithmic. *, $p < 0.05$ (mean \pm S.E.M., ANOVA); **, $p < 0.01$ (mean \pm S.E.M., ANOVA); ***, $p < 0.001$ (mean \pm S.E.M., ANOVA) of amiodarone or desethylamiodarone from control.

of 6 to 30 μM . In case of the complex I-supported respiration, above the concentration of 30 μM , desethylamiodarone presented a similar rate of inhibition that was observed with equimolar concentrations of amiodarone (Fig. 5A). However, in the case of complex II-supported respiration, above the concentration of 30 μM , desethylamiodarone presented a significantly higher rate of inhibition ($p < 0.01$) than the one observed by equimolar concentrations of amiodarone (Fig. 5B).

The effect of amiodarone or desethylamiodarone on isolated rat liver mitochondria was basically the same (data not shown).

Effect of Amiodarone and Desethylamiodarone on Permeability Transition in Isolated Mitochondria. To demonstrate the direct effect of amiodarone and desethylamiodarone on the mitochondrial permeability transition, we monitored mitochondrial swelling from isolated, Percoll gradient-purified rat liver 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+} (Fig. 6A, line 2, and B, line 2) was completely inhibited by 2.5 μM CsA (Fig. 6A, line 3, and B, line 3) or by 1 μM FCCP (data not shown). Depending on its concentration, amiodarone had a biphasic 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. 6A, lines 4–7) with the IC_{50} value of $3.9 \pm 0.8 \mu\text{M}$. The most pronounced inhibitory effect of amiodarone on the swelling induced by 60 μM Ca^{2+} was at the concentration of 10 μM (Fig. 6A, line 7). At higher concentrations, amiodarone proved to be less effective in delaying the Ca^{2+} -induced swelling (Fig. 6A, line 8). In contrast to amiodarone, desethylamiodarone did not show any inhibitory effect on the mitochondrial permeability transition induced by 60 μM Ca^{2+} up to the concentration of 10 μM (Fig. 6B, lines 4–8).

At concentrations above 10 μM , amiodarone induced mitochondrial swelling by its own (Fig. 7A, lines 5 and 7) that was not inhibited by 2.5 μM CsA (Fig. 7A, lines 6 and 8). In contrast to 30 μM amiodarone, which developed swelling with a rate significantly slower than that of the Ca^{2+} -in-

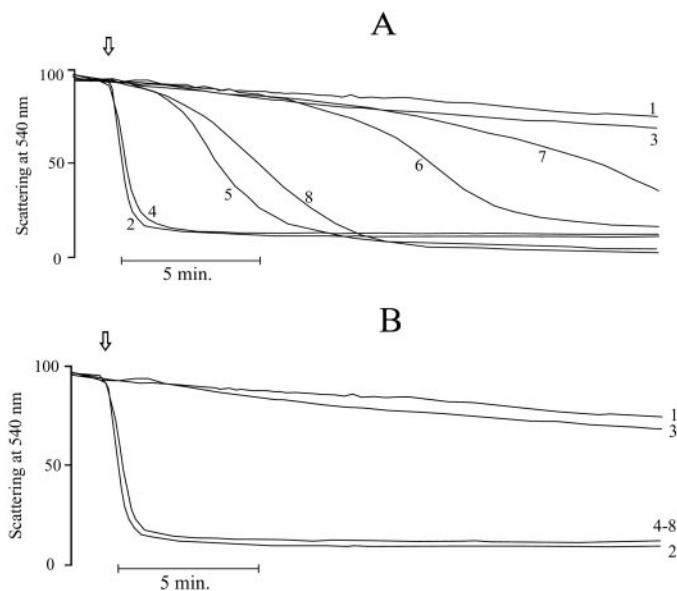


Fig. 6. Effect of amiodarone or desethylamiodarone on Ca^{2+} -induced mitochondrial swelling. Mitochondrial swelling was demonstrated by monitoring E_{540} in isolated rat liver mitochondria. Amiodarone (Ad) at the indicated concentration or 2.5 μM CsA was present throughout the experiment (A). Alternatively,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

duced swelling, desethylamiodarone, at the concentration of 30 μM , induced swelling with a rate similar to that of the Ca^{2+} -induced one (Fig. 7B, line 7). The swelling induced by desethylamiodarone was not inhibited by 2.5 μM CsA (Fig. 7B, lines 6 and 8).

The effect of amiodarone or desethylamiodarone on isolated rat heart mitochondria was basically the same (data not shown).

Effect of Amiodarone and Desethylamiodarone on Membrane Potential in Isolated Mitochondria. Ca^{2+} (60 μM) caused the dissipation of $\Delta\Psi$, as detected by the release of the membrane potential sensitive dye Rh123 from isolated liver mitochondria (Fig. 8A, line 2). When the mitochondrial membrane was depolarized by Ca^{2+} in the presence of 2.5 μM CsA, after a transient depolarization lasting for about a minute, $\Delta\Psi$ returned to the value identical to the one before the addition of Ca^{2+} (Fig. 8A, line 4). Amiodarone (10 μM) depolarized the mitochondrial membrane in a similar extent as did the 60 μM Ca^{2+} , (Fig. 8A, line 3); however, its depolarizing effect was not influenced at all by 2.5 μM CsA (Fig. 8A, line 5). Amiodarone caused a concentration-dependent release of Rh123 from liver mitochondria with a calculated EC_{50} value of $4.2 \pm 0.7 \mu\text{M}$ (Fig. 8B, lines 2–6). In contrast, desethylamiodarone, up to the concentration of 10 μM , did not induce the dissipation of $\Delta\Psi$ (Fig. 8C, lines 2–5). However, desethylamiodarone, at the concentration of 20 μM (Fig. 8C, line 6), caused Rh123 release from the isolated mitochondria as did 20 μM amiodarone (Fig. 8B, line 6) and 60 μM Ca^{2+} (Fig. 8A, line 2) or as did 1 μM FCCP (data not shown). The calculated EC_{50} value for desethylamiodarone

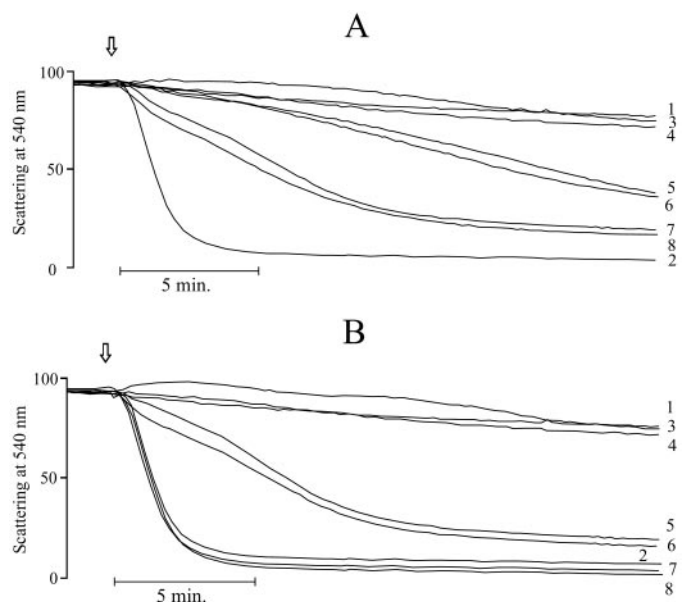


Fig. 7. Mitochondrial swelling induced by amiodarone or desethylamiodarone. Mitochondrial swelling was demonstrated by monitoring E_{540} in isolated rat liver mitochondria. CsA, at the concentration of 2.5 μM , where indicated, was present throughout the experiment. Swelling was induced by adding amiodarone (Ad) at the indicated concentration (A) or Dea at the indicated concentration (B), or 60 μM Ca^{2+} at the arrow. A, line 1, baseline swelling (no agent); line 2, 60 μM Ca^{2+} -induced swelling (no Ad or CsA); 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; and line 8, 30 μM Ad + CsA. B, line 1, baseline swelling (no agent); line 2, 60 μM Ca^{2+} -induced swelling (no Dea or CsA); line 3, 10 μM Dea; line 4, 10 μM Dea + CsA; line 5, 20 μM Dea; line 6, 20 μM Dea + CsA; line 7, 30 μM Dea; and line 8, 30 μM Dea + CsA.

was $16.3 \pm 2.3 \mu\text{M}$. The depolarizing effect of 20 μM desethylamiodarone was not influenced at all by 2.5 μM CsA (data not shown).

Effect of Amiodarone and Desethylamiodarone on Mitochondrial ROS Production. Because ROS formation can induce mitochondrial permeability transition, we studied the effect of amiodarone and desethylamiodarone 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 nonfluorescent 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, whereas Rh123 fluorescence reflects to ROS levels in aqueous phase. Amiodarone and desethylamiodarone did not induce ROS production in either case in the concentration range from 1 to 100 μM (data not shown).

Discussion

Both amiodarone and desethylamiodarone have damaging effect on extracardiac tissues as lung, thyroid, liver, and pancreas (Amico et al., 1984; Martin and Howard, 1985; Card et al., 1998). Whereas desethylamiodarone has similar antiarrhythmic activity as amiodarone (Pallandi and Campbell, 1987), their effect on postischemic heart has not been compared. To assess this issue, we monitored real-time in situ concentrations of ATP, creatine phosphate, and inorganic phosphate during ischemia-reperfusion of Langendorff-per-

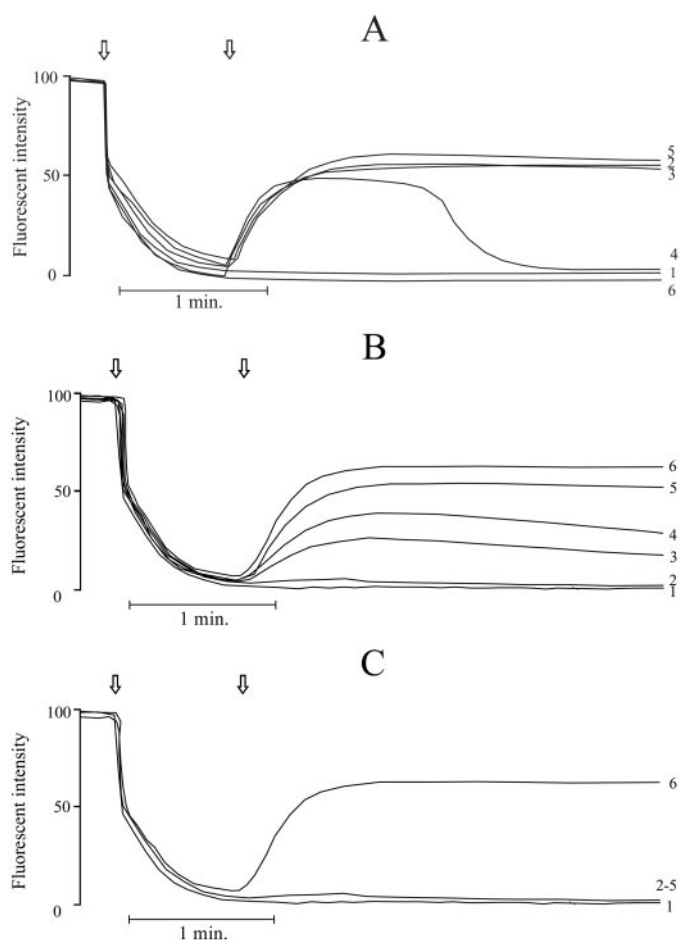


Fig. 8. Effect of amiodarone or desethylamiodarone on the mitochondrial membrane potential in isolated mitochondria. Membrane potential was monitored by measuring the fluorescence intensity of the cationic fluorescent dye rhodamine 123. Isolated rat liver mitochondria, added at the first arrow, take up the dye in a voltage-dependent manner and quench fluorescence. Amiodarone (Ad) and Dea at the concentrations indicated or $60 \mu\text{M}$ Ca^{2+} (either added at second arrow) induces depolarization, resulting in release of the dye and increase of the fluorescence intensity. A, line 1, no agent; line 2, Ca^{2+} ; line 3, $10 \mu\text{M}$ Ad; line 4, $\text{Ca}^{2+} + 2.5 \mu\text{M}$ CsA; line 5, $10 \mu\text{M}$ Ad + $2.5 \mu\text{M}$ CsA; and line 6, $10 \mu\text{M}$ Dea. B, line 1, no agent; line 2, $1 \mu\text{M}$ Ad; line 3, $2.5 \mu\text{M}$ Ad; line 4, $5 \mu\text{M}$ Ad; line 5, $10 \mu\text{M}$ Ad; and line 6, $20 \mu\text{M}$ Ad. C, line 1, no agent; line 2, $1 \mu\text{M}$ Dea; line 3, $2.5 \mu\text{M}$ Dea; line 4, $5 \mu\text{M}$ Dea; line 5, $10 \mu\text{M}$ Dea; and line 6, $20 \mu\text{M}$ Dea.

fused rat hearts by ^{31}P NMR spectroscopy. A single i.v. injection of 20 mg/kg amiodarone or desethylamiodarone administered 30 min before the removal of the heart resulted in a rather low blood concentration of the drugs, whereas their concentrations in the heart mitochondria were 3.5 and $4.1 \mu\text{M}$ for amiodarone and desethylamiodarone, respectively. Under these conditions, amiodarone had a significant protecting effect on the energy metabolism of perfused hearts during ischemia-reperfusion indicated by the faster and more complete recovery of the high-energy phosphate concentrations. However, in the case of desethylamiodarone, a similar dose and timing of pretreatment had no effect on the concentrations of creatine phosphate, ATP and inorganic phosphate during ischemia-reperfusion, when compared with the control (Fig. 1, A–C). These indicate that desethylamiodarone does not contribute to amiodarone's cardioprotective effect.

To assess cytotoxicity of both amiodarone and desethylamiodarone on cardiomyocytes and extracardiac cells, we se-

lected cell lines with comparable doubling time, originating from extracardiac tissues exhibiting an increased vulnerability toward amiodarone administration, namely, normal liver and pancreas cancer cells. The effect of the drugs on thyroid cells has been reported previously (Di Matola et al., 2000), whereas normal pancreatic as well as nonfibroblast lung cell lines are not available. We performed viability studies using the mitochondrial dye MTT^+ . The water-insoluble blue formazan dye reflects not only the number of viable cells but also the number and respiratory function of mitochondria. This can explain the moderate increase of the optical density detected in the presence of low concentrations (10 – $20 \mu\text{M}$) of amiodarone compared with the control in H9C2 cardiomyocytes (Fig. 3A), which is due to the uncoupling effect of the drug, rather than the increase in the number of cells. This is more pronounced in the case when H9C2 or WRL-68 cell lines were exposed to amiodarone, desethylamiodarone, and FCCP for only 1 h (Fig. 4, A and B). Amiodarone exhibited an uncoupling effect similar to FCCP up to the concentration of $30 \mu\text{M}$, in H9C2 but not in WRL-68 cells, whereas desethylamiodarone lacked the uncoupling effect, presenting inhibition in both cell lines above the concentration of $10 \mu\text{M}$. In each of the cell lines examined, desethylamiodarone proved to be significantly more toxic than the equimolar concentrations of amiodarone in a specific concentration range (Fig. 3, A–D). This is in accord with the results of other reports (Daniels et al., 1989; Ogle and Reasor, 1990; Di Matola et al., 2000; Bolt et al., 2001). Extracardiac cell lines exhibited a marked sensitivity toward amiodarone toxicity in the whole concentration range tested compared with the toxicity in H9C2 cell line, as demonstrated by their lower LD_{50} values. Desethylamiodarone also was more toxic in case of the BRL-3A and WRL-68 cells, than in H9C2 cardiomyocytes, but not in the case of the pancreas cell line, which presented a similar LD_{50} value for desethylamiodarone as did the cardiomyocytes (Table 2). PANC-1 cell line is however a carcinoma line, representing a more immature state of differentiation than the liver cells, which could explain its decreased susceptibility toward the drug, indicated by a higher LD_{50} value. The examined concentration range of 1 to $100 \mu\text{M}$ is in accord with the levels of either amiodarone or desethylamiodarone observed in different tissues after long-term amiodarone treatment. These results suggest that desethylamiodarone is unlikely to contribute to the cardioprotective effect of its parent drug.

Although the molecular mechanism of the antiarrhythmic effect of amiodarone is well established (Singh and Vaughan Williams, 1970; Nattel et al., 1987), the mechanism of cardioprotectivity and cytotoxicity is obscure. The observation that amiodarone protected the energy metabolism in perfused hearts suggested that the cardioprotectivity of the drug could be consequence of its direct mitochondrial effect, by influencing $\Delta\Psi$, ROS production, respiration, or permeability transition. Although some studies addressed the effect of amiodarone and desethylamiodarone on mitochondrial respiration and ROS production (Fromenty et al., 1990; Di Matola et al., 2000), the effect of the drugs on permeability transition has not been fully disclosed (Varbiro et al., 2003), although the involvement of permeability transition in the collapse of oxidative phosphorylation and ion homeostasis, as well as in mediation of both necrotic and apoptotic cell death is well established (Kroemer and Reed, 2000). Therefore, we studied

the direct effect of the drugs on isolated, Percoll-gradient purified mitochondria from rat liver and heart, as well as the effect of amiodarone on the release of AIF from mitochondria and its translocation to the nucleus in perfused rat hearts.

We revealed that amiodarone has a mixed effect on mitochondrial functions. In low concentration range, where it exhibited cardioprotective effect and inhibited the release of AIF from the mitochondria in perfused hearts, it stimulated state 4 respiration due to an uncoupling effect and inhibited the Ca^{2+} -induced mitochondrial swelling, whereas it caused a moderate dissipation of the $\Delta\Psi$. However, at higher concentrations it exerted an inhibitory effect on the mitochondrial respiration and simultaneously induced a mitochondrial swelling that was not inhibited by CsA. In contrast, desethylamiodarone did not stimulate state 4 respiration, did not inhibit the Ca^{2+} -induced mitochondrial permeability transition, and did not induce the collapse of $\Delta\Psi$ in low concentrations, and it did not prevent the release and nuclear translocation of AIF in perfused rat hearts. At higher concentrations, similar to amiodarone, it induced a mitochondrial swelling that was not inhibited by CsA and inhibited respiration. This inhibitory effect was more prominent on complex II-supported respiration than that of amiodarone (Bolt et al., 2001).

The mitochondrial permeability transition after the opening of the permeability transition pore causes swelling of the matrix, leading to membrane disruption and finally cell death, whereas the release of mitochondrial proteins such as cytochrome *c* or AIF lead to the activation of the apoptotic pathway (Green and Reed, 1998; Kroemer and Reed, 2000). Although the dissipation of $\Delta\Psi$ was previously suspected to be a phenomenon tightly associated with permeability transition, recent reports have revealed, the collapse of the mitochondrial membrane potential does not induce, but rather prevents, mitochondrial swelling (Aronis et al., 2002; Kahlert and Reiser, 2002). We also found that FCCP, a widely used uncoupling agent, inhibits the Ca^{2+} -induced mitochondrial swelling, whereas it dissipated $\Delta\Psi$. Amiodarone, similarly to FCCP, exhibits uncoupling effect, inhibits swelling, and dissipates $\Delta\Psi$. A theory suggests that during uncoupling, the mitochondrial respiratory chain works more efficiently, leading to less leakage of electrons and thus to lower levels of ROS generation (Budd et al., 1997), obscuring the mechanism by which FCCP or lower membrane potential promotes pore opening. Previous reports (Ribeiro et al., 1997; Di Matola et al., 2000), and our findings that amiodarone does not induce ROS production in isolated mitochondria is also in accord with this theory. In the case of desethylamiodarone, however, none of the above-described effects of amiodarone were found; moreover, it was reported to increase the intracellular cytosolic free Ca^{2+} concentration (Himmel et al., 2000), which could also contribute to its enhanced toxicity.

In conclusion, we present clear evidence that amiodarone but not desethylamiodarone protects the mitochondrial energy metabolism of the perfused heart during ischemia and reperfusion as detected by real-time in situ ^{31}P NMR measurement. We demonstrate for the first time that amiodarone prevents the mitochondrial AIF release induced by ischemia-reperfusion in perfused hearts. We also demonstrated that cardiac and extracardiac cells are more susceptible to desethylamiodarone than amiodarone. We also report that amiodarone exerts a biphasic effect on the mitochondria, with

protective effects in lower concentration and toxic properties manifesting when present at higher concentrations, whereas desethylamiodarone does not have this dual feature. Amiodarone when present in low concentrations protects the energy mechanism of postischemic heart by inhibiting the mitochondrial permeability transition and by attenuating the ROS generation. These properties of amiodarone are due to the presence of its ethyl group, because its major metabolite, desethylamiodarone, does not exhibit the cardioprotective and beneficial mitochondrial features of the parent drug. In higher concentrations, amiodarone as well as desethylamiodarone, besides inhibiting the mitochondrial respiration, can induce a CsA-independent mitochondrial swelling, thus contributing to the toxic property of the drug. Although both amiodarone and desethylamiodarone have similar antiarrhythmic properties, only amiodarone possesses a cardioprotective effect, and the frequently manifesting side effects during long-term amiodarone therapy could be related, at least in part, to the accumulation of desethylamiodarone.

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