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Protective effect of darodipine, a calcium antagonist, on rat cardiomyocytes against oxygen radical-mediated injury

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1 We used electrophysiological and electron spin resonance (e.s.r.) techniques to study the mechanism of the protective effect of darodipine on rat isolated cardiomyocytes exposed to an exogenous source of oxygen free radicals (OFR).

2 The L-type calcium current ($I_{Ca,L}$), action potential and cell shortening were measured in patch-clamped cells in the whole-cell configuration. $I_{Ca,L}$ blockade by darodipine was concentration-dependent, peak current being reduced by 20% with 50 nM and by 58% with 100 nM darodipine. The lowest concentration of darodipine did not affect action potential or twitch profile.

3 Exposure to OFR-generating solution (5 mM dihydroxyfumarate, DHF) caused the appearance of electrophysiological alterations and/or spontaneous activity in 73% of cells ($n=26$) within 5 min; action potential duration (APD) was prolonged (195 ± 16 ms vs 140 ± 6 ms in the control) and maximum diastolic potential (MDP) was reduced (-59.5 ± 2.6 mV vs -69.8 ± 0.8 mV in the control) ($P<0.05$, $n=25$).

4 A 2 min pretreatment with 50 nM darodipine significantly reduced the incidence of these arrhythmogenic events following a 5 min exposure to OFR (36% of cells, $n=14$; $P<0.05$ vs nonpretreated cells). Pretreatment with darodipine also prevented APD prolongation caused by OFR (137 ± 12 ms after DHF vs 117 ± 6 ms before DHF $n=14$, not significant) but not the decrease of MDP (-63.4 ± 2.5 mV after DHF vs -70.9 ± 1.0 mV before DHF, $P<0.05$).

5 The e.s.r. spectra obtained from the DHF-DMPO solution in the absence of darodipine demonstrated the presence of two components corresponding to two DMPO adducts. The addition of darodipine (50–500 nM) led to a concentration-dependent decrease in intensity of the signals, the intensity of the DMPO-$COO^{\cdot -}$ adduct being decreased more than that of the DMPO-$OH^{\cdot}$ adduct.

6 Our results demonstrate that darodipine dose-depentently blocks $I_{Ca,L}$ in rat isolated cardiomyocytes. Furthermore it exerts protective effects against free-radical-induced electrophysiological alterations independently of its calcium antagonistic properties; this effect is possibly due to trapping of specific radical species.

Keywords: Darodipine; oxygen radicals; cardiomyocytes; electrophysiology; L-type calcium current; electron spin resonance

Introduction

Oxygen radicals generated at the time of reflow of the ischaemic heart are responsible for some of the deleterious effects associated with reperfusion, including ventricular arrhythmias and stunning (see Hearse, 1992 for a review). We recently showed that oxygen radicals cause electrophysiological alterations in guinea-pig (Cerbai *et al*., 1991; Guerra *et al*., 1996) and rat (Mugelli *et al*., 1995) isolated cardiomyocytes. These alterations occur with a time course consistent with the rapid onset of ventricular arrhythmias in the reperfused heart (Cerbai *et al*., 1991; 1992). Alterations include a marked prolongation of the action potential duration and induction of early and delayed after-depolarizations leading to abnormal activity. The underlying ionic mechanisms are likely to be a reduction of outward potassium currents and calcium overload (Cerbai *et al*., 1991; Jabr & Cole, 1993; Mugelli *et al*., 1995). Calcium overload does not appear to be due to an increased calcium entry through calcium channels, since L-type calcium current ($I_{Ca,L}$) is actually decreased by free radicals (Cerbai *et al*., 1991; Guerra *et al*., 1996). The decrease in $I_{Ca,L}$ is paralleled by a decrease in dihydropyridine binding sites (Guerra *et al*., 1996).

Some blockers of β-adrenoceptors and calcium antagonists (especially of the dihydropyridine type) have been recently shown to inhibit membrane lipid peroxidation initiated by free radical generating systems (Janero & Burghardt, 1989; Janero *et al*., 1988; Mak & Weglicki, 1990; Mak *et al*., 1988; Van Amsterdam *et al*., 1992; Yue *et al*., 1992). It has also been observed that nicardipine (but not nifedipine) has a direct protective effect on rat single, cardiac myocytes exposed to reoxygenation injury (Hano *et al*., 1991). This effect appears to be independent on the L-type channel blocking properties of nicardipine. The basis of these protective actions are not known. However, it seems that high lipophilicity and affinity for the dihydropyridine calcium channel receptor are obligatory prerequisites.

Darodipine (PY 108-068) exhibits higher solubility in organic solvents and lower aqueous solubility than the analogous compound isradipine; it rapidly binds to cellular membranes (Urien *et al*., 1987) and has a high affinity for a calcium channel binding site (Supavilai & Karobath, 1984). The drug is neutral at physiological pH and light stable (Hof, 1985).

Given these premises, we thought it of interest to evaluate if darodipine has protective effects against free-radical induced electrophysiological alterations at concentrations exerting a minimal calcium antagonistic effect. In the present study we briefly describe the effects of darodipine on $I_{Ca,L}$ in rat ventricular myocytes and then we demonstrated its protective actions on the electrophysiological changes produced by free radicals in rat ventricular myocytes.

To gain insight into the mechanism of this protective action, the direct scavenger properties of darodipine were evaluated by

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means of the spin trap method (e.s.r.) (Matucci *et al*., 1996); by analysing the e.s.r. spectra, this method allows the identification of the radical species formed in the system and an evaluation of the relative amount of the radical removed by darodipine.

Methods

Cell isolation

The investigation conforms to the rules for the care and use of laboratory animals of the European Community (86/609/CEE).

Single left ventricular myocytes were isolated from adult male rats (Wistar, 200–300 g) by use of a protocol based on previously described procedures (Cerbai *et al*., 1995). After the rat had been killed the heart was rapidly excised, mounted in a Langendorff apparatus and then retrogradely perfused for 5 min with a low-calcium solution (LCS) of the following composition (mM): NaCl 120, KCl 10, KH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 10, taurine 20, pyruvate 5, pH 7.2 with HEPES/NaOH. LCS was thermostated at 37°C and equilibrated with 100% O_2. The solution was then quickly changed to LCS plus 1 mg ml^{-1} collagenase (Type I, Sigma), 0.03 mg ml^{-1} dispase (Boehringer), 1 mg ml^{-1} albumin (Serva) for 10 min. Ventricles were removed with fine scissors, cut into chunks which were stirred in the LCS. Cardiomyocytes that appeared in the supernatant were concentrated by gravity sedimentation, collected and stored in LCS at room temperature. Cells were kept in LCS supplemented with 1 mM $CaCl_2$, penicillin (50 iu ml^{-1}) and streptomycin (50 μg ml^{-1}) (Gibco), and used within 10 h of their isolation.

Electrophysiological experiments

The experimental set-up was similar to that described in Cerbai *et al*. (1995). A drop of cells was placed in the experimental chamber (0.2 ml) mounted on the plate of an inverted microscope (TMS, Nikon), connected to a monitor (Hitachi VM 122) via a camera (Hitachi HM30). The whole-cell configuration of the patch-clamp technique was used to record action potentials and membrane currents. The electrical signal was recorded by a patch amplifier (Axopatch 1D, Axon Instrument Inc.), digitized (Labmaster TL-1 DMA, Scientific Solutions), and displayed on the monitor of a personal computer and a digital oscilloscope (Nicolet 310, Nicolet Instrumentation Company). The cut-off frequency was 20 kHz. Current and voltage protocol generation, data acquisition and analysis were performed by use of the pClamp software (Version 5.5.1, Axon Instrument Inc.). MicroCal Origin (MicroCal Software Inc.) was used for further analysis.

Recording was started after 5 min dialysis of the cell. Action potentials were elicited at a rate of 0.2 Hz and sampled at 1 kHz. Cell length (Ruocco *et al*., 1996) was measured by means of a Video Dimension Analyzer (Instrumentation for Physiology and Medicine, Inc.) connected to the microscope via a video camera (JVC). The marks appearing on a monitor were positioned at one end of the cell and adjusted in order to have a stable signal. The signal was amplified (100×), digitized by the A/D converter, sampled at 1 kHz and recorded simultaneously with the electrophysiological signal from the patch-clamped myocyte. With this system, the measurement of cell shortening is delayed in comparison with the recording of the action potential; the delay is approximately 40 ms when compared with cell shortening obtained with the photodiode system (Spurgeon *et al*., 1990). Cell shortening traces have been corrected for this delay. Measurements of cell length are correlated with changes in intracellular calcium concentration, as demonstrated by simultaneous recordings of intracellular calcium activity and sarcomere length (Spurgeon *et al*., 1990). However, in our experimental conditions and for our purposes, changes in cell length were considered only as a rough index of cell contractility, and no attempt was made to use them as a quantitative measurement of cell shortening; therefore, they are expressed in arbitrary units (AU).

$I_{Ca,L}$ was elicited by 200 ms depolarizing steps to 0 mV from a holding potential of −70 mV (unless indicated), preceded by a 20 ms step to −40 mV to inactivate sodium current. Steps were applied at low frequency (0.2 Hz) and sampled at 5 kHz. $I_{Ca,L}$ amplitude was measured as the difference between steady state current, measured at the end of the depolarizing step at 0 mV, and peak inward current. To minimize the amplitude of the capacitative transient, series resistance was compensated by 80% and membrane capacitance was corrected up to 100 pF.

Solutions

Cells were superfused by means of a peristaltic pump (Masterflex model 7524/05, Cole-Parmer Instrument Company) at a flow rate of 1.8 ml min^{-1}, a three-line system controlled by electronic valves allowed solutions to be changed rapidly. For $I_{Ca,L}$ measurements both extracellular and pipette solutions contained Cs^+, in order to block inward and outward potassium currents. The composition of the bath solution was (mM): NaCl 137, CsCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.2, glucose 10, HEPES 5, pH was adjusted to 7.35 with NaOH, the temperature was kept at 36±0.5°C. Patch pipettes (Corning Capillaries 7052, Garner Glass), having a resistance of 1.5–2 MΩ, were pulled by means of a two-step puller (Hans Otchozki, Homburg), fire polished and filled with a solution of the following composition (mM): CsCl 140, $MgCl_2$ 1, Na_2-ATP 5, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′N′-tetra acetic acid (EGTA) 1, HEPES 10, adjusted to pH 7.20 with KOH. No correction was made for liquid junction potential between internal pipette solution and bath solutions. To measure action potential, Cs^+ was replaced with K^+ in all solutions. In the experiments designed to study the effects of oxygen free radicals (OFR) on the electrophysiological activity of rat myocytes, we used pipette solution having a lower EGTA concentration (50 μM), high enough to avoid calcium loading through the electrode, but not sufficient to chelate $[Ca]_i$, which consequently could change freely (Guerra *et al*., 1996).

The OFR-generating solution consisted of 5 mM dihydroxyfumarate (DHF), added to the Tyrode solution just before use. Both control and DHF-containing solution were oxygenated with 100% O_2. Under our experimental conditions of pH and oxygen tension, DHF underwent redox cycling, with formation of superoxide radicals and hydrogen peroxide (Mak *et al*., 1983; Kramer *et al*., 1984). Darodipine was diluted in ethanol and then in Tyrode to a final concentration of 50 or 100 nM. At the concentration used (less than 0.1%), ethanol was devoid of direct electrophysiological effect on action potential and membrane currents.

E.s.r. experiments

The e.s.r. spectra of the adducts formed by DMPO and the radicals produced by the system (termed DMPO-radical) were composed of multiplets of derivative lines due to the coupling between the unpaired electron spin (S = 1/2) and the nuclear spins of the nitrogen nucleus (I = 1) of the DMPO-radical and of the surrounding protons (I = 1/2) in the radical structure. Each group of magnetically equivalent protons gave rise to a multiplet ($n+1$, where n is the number of protons) of lines in the e.s.r. spectra, and the hyperfine coupling constants (A_i) were affected by the overall structure of the radical. The number of lines, their relative intensities and the A_i values were therefore distinctive of a type of radical. In the present case, identification of the type of radical responsible for each e.s.r. signal was achieved by evaluating the hyperfine coupling constants A_N and $A_H{}^{\beta}$, and by referring to data presented in the literature on the coupling constants for the different DMPO adducts.

For a correct evaluation of the magnetic parameters, the spectra were simulated as a combination of lorentzian lines (the multiplets of lines arising from the hyperfine coupling). Integration of the adsorption signals allowed for the evaluation of the relative intensities of the spectra.

Usually DMPO produced radical species by aging or light exposure. In any case the signal from the water solution of DMPO was subtracted from the signals of the samples under study, recorded in the same experimental conditions.

The e.s.r. spectra were recorded with the aid of a Bruker 200D spectrometer working at X-band of about 9.5 GHz and interfaced with a Stelar software which also allowed data handling.

Darodipine was tested as radical scavenger against DMPO, with an oxygenated Tyrode solution containing 5 mM DHF (see above) as a source of radicals. In the e.s.r. experiments the compounds were added as follows: DMPO 0.1 M, DHF 5 mM and control Tyrode solution or Tyrode plus darodipine (50–500 nM) to the sample. The samples were mixed, pipetted into a flat cell for e.s.r. and the e.s.r. spectra were recorded at room temperature.

The relative intensities of the e.s.r. spectral components, arising from different DMPO-radical adducts, were evaluated with the aid of the flat cell inserted and fixed into the e.s.r. cavity. Each solution to be analysed was flowed in the flat cell; any measurement was repeated to control the reproducibility of the results. Aging of the DHF solution in the presence of DMPO corresponded to the increase in intensity of the DMPO-$COO^{\cdot -}$ adduct at the expenses of the DMPO-$OH^{\cdot}$ adduct (results not shown). To compare the results correctly from different preparations, all the e.s.r. measurements were performed by using freshly prepared DHF solutions. At least 2–3 min were necessary to fill the solution into the flat cell and to set the instrument for signal registration; all signals were recorded 5 min after sample preparation.

Electrophysiological data analysis

The occurrence of oxygen free radical (OFR)-induced spontaneous activity in the absence and presence of darodipine was compared by means of the χ^2 test (2×2 contingency table). All the other data are presented as the mean $\pm$ s.e.mean. Comparison between two groups was performed by means of Student's t test for paired or grouped data. A P value of less than 0.05 was considered significant.

Results

Electrophysiological characterization of darodipine

Figure 1 shows the time course of the effect of 100 nM darodipine on $I_{Ca,L}$ elicited by a 200 ms pulse (preceded by a 20 ms prestep to -40 mV) to 0 mV from a holding potential of -70 mV. Peak $I_{Ca,L}$ decreased gradually, reaching a steady-state within 2 min. In 6 different cells, the mean reduction was 58% of the pre-drug value. After 3–4 min of wash-out, a significant recovery of current amplitude was observed. Figure 1B shows representative current traces recorded at different times as indicated by the letters: while peak amplitude is clearly modified by darodipine and wash-out, no obvious effect on time to peak and inactivation kinetics of $I_{Ca,L}$ is apparent.

As expected for a dihydropyridine compound, the calcium-antagonistic activity of darodipine was voltage-dependent. This is shown in Figure 2: in (a), the $I_{Ca,L}$ peak is presented as a function of time before and after 100 nM darodipine. In the control, changing the holding potential (HP) from -70 mV to -40 mV typically induced a 15–18% reduction of $I_{Ca,L}$ amplitude (Cerbai *et al*., 1997a,b); the effect could be completely reversed upon return to the more negative HP. Darodipine caused a progressive decrease of $I_{Ca,L}$ to a new steady state value. Switching the HP from -70 to -40 mV led to a rapid 'extra' block of calcium channels. The voltage-dependent

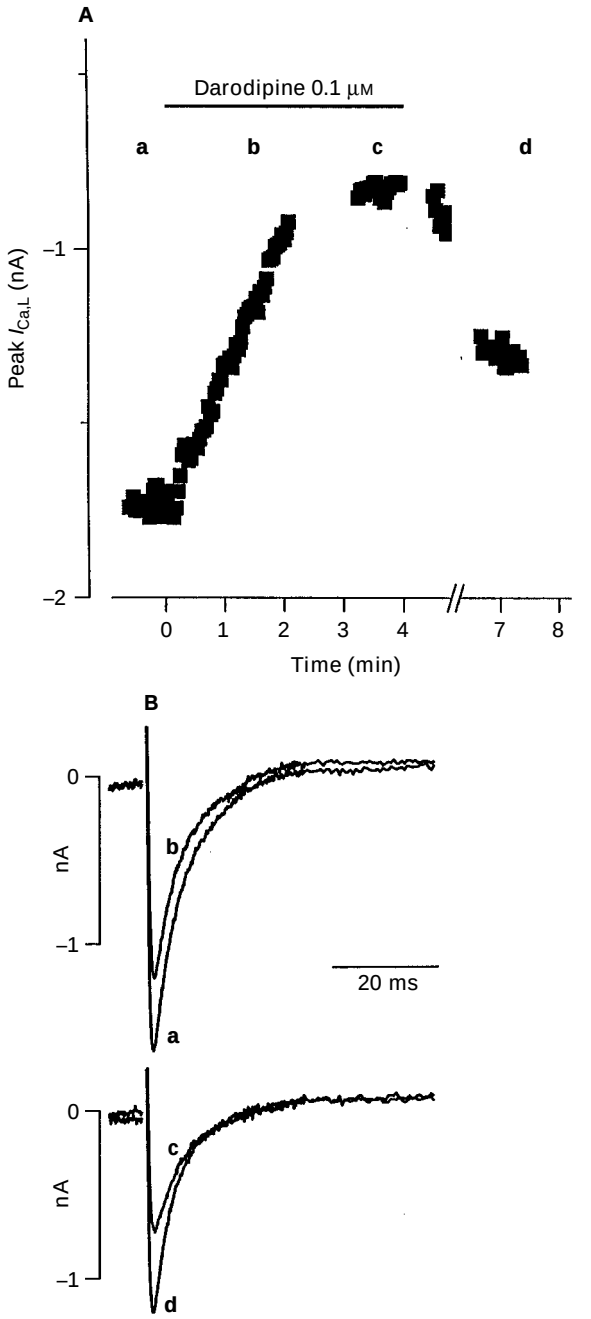

Figure 1 (A) Time-course of the effect of darodipine (100 nM) on peak $I_{Ca,L}$. The duration of the superfusion with darodipine is indicated by the line. (B) Shows current recordings obtained at the time indicated by the corresponding letter in (A).

block, induced at depolarized HP, was rapidly and completely reversed upon return to -70 mV, as previously described in the control. However, the effect was quantitatively more pronounced in the presence than in the absence of the drug. (b) Shows superimposed current traces recorded in the presence of darodipine at the first, second and tenth voltage-step elicited from HP of -40 mV (upper traces), and those recorded during the steps immediately after return to -70 mV (bottom traces).

Darodipine and OFR-mediated electrophysiological alterations

The protective action by darodipine on OFR-mediated electrophysiological alterations was tested at a lower drug concentration (50 nM), that is, a concentration with low calcium antagonistic activity and devoid of discernible effects on elec-

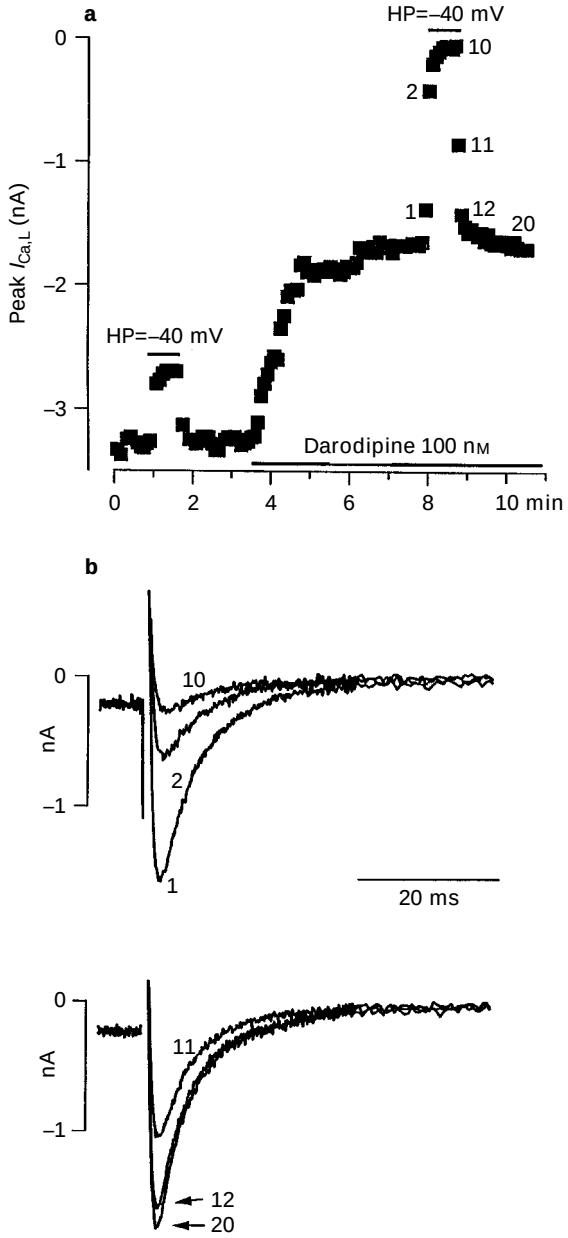

Figure 2 Voltage-dependent block of $I_{Ca,L}$ by darodipine. (a) Plot of the peak current amplitude before and after superfusion with darodipine (indicated by the solid line). When indicated, the holding potential (HP) was changed from −70 mV to −40 mV. (b) Current recordings obtained in the presence of darodipine at the time indicated by the corresponding number.

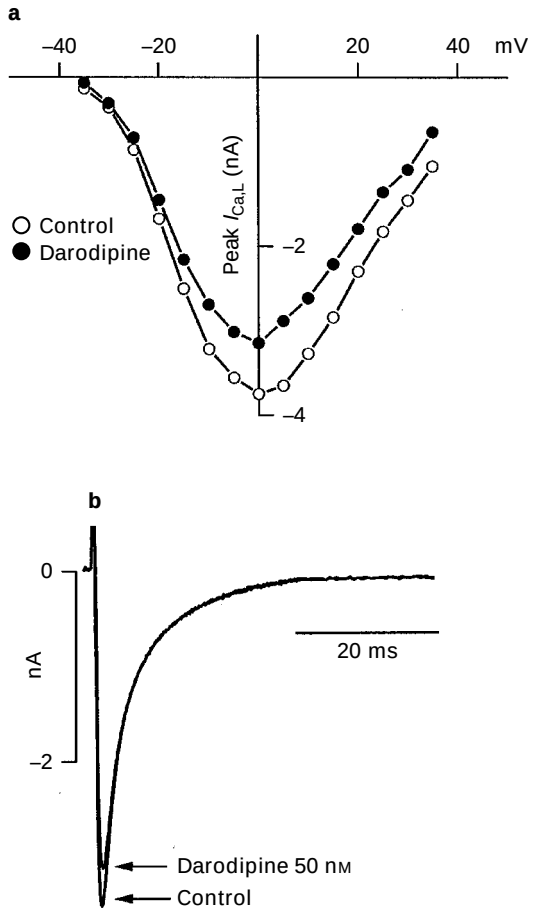

Figure 3 (a) Typical current-voltage relationship for $I_{Ca,L}$ obtained in the absence (control) and presence of 50 nM darodipine. (b) Superimposed current traces recorded during a voltage step to 0 mV, in the absence and in the presence of darodipine.

trical and mechanical activities when recorded at the same stimulation rate as used for voltage clamp experiments. Figure 3 shows a typical current-voltage (*I*-V) relationship for $I_{Ca,L}$ in the absence and presence of 50 nM darodipine (a), and the corresponding current traces recorded during the step at 0 mV (b): it is apparent that 50 nM darodipine caused a small reduction in $I_{Ca,L}$ amplitude (to 76% of the control value, $n=5$) without modifying the *I*-V shape. As shown in Figure 4 this concentration of darodipine did not significantly modify the action potential profile nor the corresponding 'twitch' after 4 min. The lack of effect of darodipine on the repolarization phase is consistent with the absence of effects on K^+ repolarizing currents (data not shown).

Exposure of myocytes to the OFR-generating solution (5 mM DHF in the presence of O_2) caused, as expected (Cerbai *et al*., 1991; Jabr & Cole, 1993; 1995; Mugelli *et al*., 1995; Guerra *et al*., 1996), the appearance of a variety of electrophysiological alterations. A typical experiment is shown in Figure 5: (a) shows the control action potential (upper trace) and the associated twitch (bottom trace). After 1 min exposure to DHF (b) the repolarization phase is prolonged by 30 ms and a spontaneous oscillation of the membrane potential, accompanied by a simultaneous change of cell length (aftercontraction, a well known sign of intracellular calcium overload), appears during the diastolic phase. After 3 min (c) the resting potential is markedly depolarized and the twitch amplitude associated with the driven AP is further reduced. A decrease in contractility in the presence of calcium overload has been previously shown to occur in trabeculae of the right ventricle of the rat heart exposed to exogenous source of oxygen free radicals (Gao *et al*., 1996). Spontaneous abnormal action potentials appear after the driven AP, showing transient depolarization during the early phase of repolarization (early afterdepolarizations) and, at the same time, typical 'multipeak' contractions. Early-afterdepolarizations and/or spontaneous activity appeared within 5 min in the majority of the cells (73%, $n=26$). A summary of the changes in action potential duration (APD) and maximum diastolic potential (MDP) observed during exposure to OFR is presented in Figure 6. APD measured at 90% repolarization (APD_{90}) was significantly increased from 140 ± 6 ms in the control to 195 ± 16 ms after 3 min exposure to DHF ($P<0.01$, $n=25$) (Figure 6a, left). MDP was reduced from -69.8 ± 0.8 mV in the control to -59.5 ± 2.6 mV in DHF ($P<0.05$, $n=25$; Figure 6b, left). A 2 min pretreatment with 50 nM darodipine prevented the prolongation of APD induced by 5 min exposure to the OFR-generating solution (Figure 6a right). APD_{90} in the presence of DHF and darodipine (137 ± 12 ms, $n=14$) was not significantly different from that measured in the same

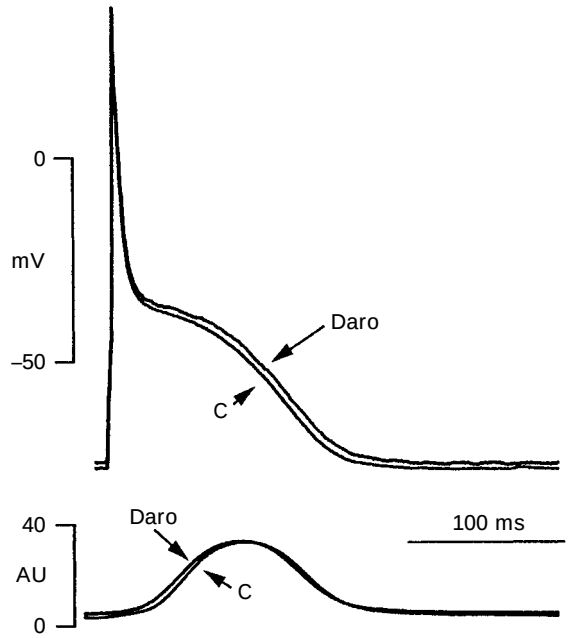

Figure 4 Superimposed action potentials (upper traces) and cell shortening (bottom traces) obtained from a single rat myocyte before (C) and after 4 min superfusion with 50 nM darodipine (Daro).

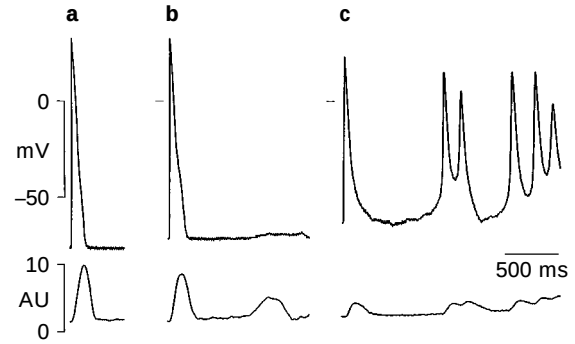

Figure 5 Typical experiment showing the effect of oxygen radicals on the electrical activity (upper traces) and contractility (bottom traces) of a single rat myocyte. Traces were recorded before (a) and after 1 min (b) and 3 min (c) superfusion with DHF.

cells superfused for 2 min with darodipine before exposure to DHF (117 ± 6 ms) or in the control (117 ± 7 ms), but was significantly shorter than in unpretreated cells ($n = 25$, $P < 0.05$). However, 2 min pretreatment with the drug could only partially prevent the depolarization induced by free radicals (Figure 6b). In the control, MDP in the absence or in the presence of darodipine was -72.3 ± 0.8 mV and -70.9 ± 1.0 mV ($n = 14$), respectively; these values were significantly different ($P < 0.05$) from that measured after DHF (-63.4 ± 2.5 mV). Interestingly, arrhythmogenic alterations due to exposure to OFR appeared in only 36% of cells ($n = 14$), a result significantly different ($P < 0.05$) from that observed in cells not pretreated with darodipine. Figure 7 shows a typical recording obtained from a patch-clamped myocyte in which both the electrical activity (upper traces) and cell length (bottom traces) were measured during such an experiment. As already shown, after 2 min superfusion with 50 nM darodipine (b) both AP and twitch profile were similar to that recorded in the control (a). Exposure to DHF for 3 min (c) only caused a slight depolarization of the resting potential, and a slight decrease of the amplitude of cell shortening. A longer exposure to OFR (5 min, d) caused a lengthening of APD and a more evident reduction of MDP, accompanied by a decrease of the

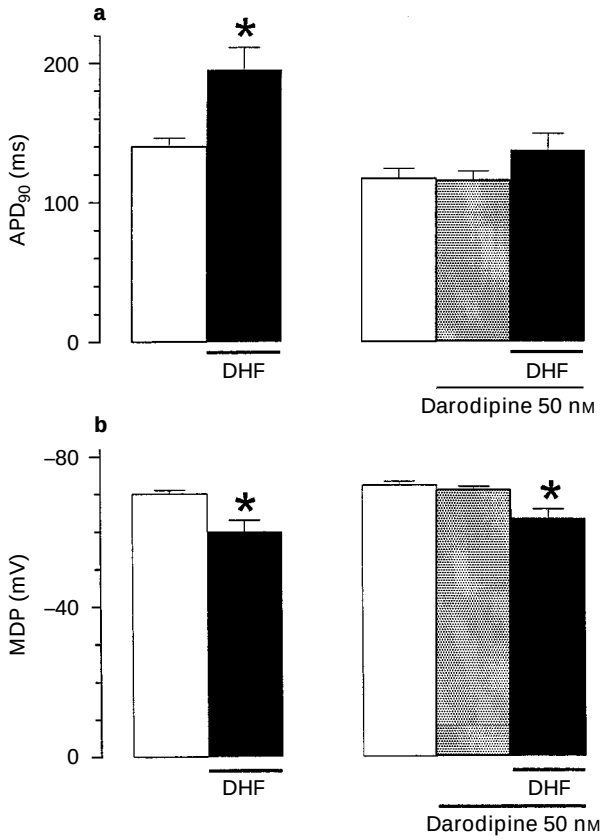

Figure 6 Summary of the effects of 5 min exposure to oxygen radicals on action potential duration (APD_{90}) and maximum diastolic potential (MDP) in control cells (left) and in cells pretreated with darodipine (right). Open columns: control; solid columns: DHF 5 mM; stippled columns: darodipine. $^*P < 0.05$ vs respective controls.

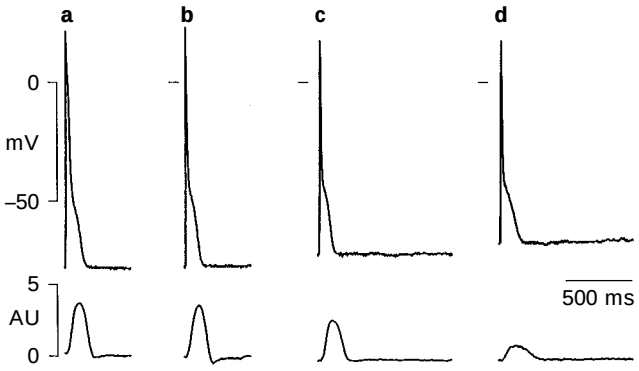

Figure 7 Typical experiment showing the protective effect of darodipine against the damage by oxygen radicals. Each panel shows the electrical activity (upper trace) and contractility (bottom trace) of a single rat myocyte in control (a), after 2 min superfusion with 50 nM darodipine (b) and after 3 min (c) and 5 min (d) exposure to DHF in the continuous presence of darodipine.

twitch. However, it failed to induce the appearance of spontaneous activity or other arrhythmogenic alterations, as observed in cells not treated with darodipine (see Figure 5).

E.s.r. measurement of radical production in the absence and presence of darodipine

Figure 8 shows the e.s.r. spectra obtained from the solution of DHF in buffer containing DMPO. The spectrum (a) indicated in the figure was recorded from the DHF-DMPO solution in the absence of darodipine, whereas spectra (b) and (c) were obtained after the addition of darodipine at 50 nM and

500 nM, respectively. The spectra are well reproducible under these experimental conditions, thus ensuring the reliability of the data.

Analysis of the spectra in Figure 8 demonstrates the presence of two components, corresponding to two DMPO adducts, the hyperfine lines of which are superimposed to give the overall e.s.r. signal. The sets of hyperfine lines belonging to the two components are indicated in Figure 8 and labelled as components A and B, respectively.

The evaluation of the hyperfine coupling constants allowed the identification of the following DMPO adducts (Finkelstein *et al*., 1980): (A) DMPO-$OH^{\cdot}$:$A_N = A_H^{\beta} = 14.7$ G; (B) DMPO-$COO^{\cdot -}$:$A_N = 15.6$ G; $A_H^{\beta} = 18.8$ G.

As described in Methods, all the signals were recorded 5 min after preparation of the samples, when the components A and B in the absence of darodipine were comparable in intensity (see spectrum (a) in Figure 8). Thus, it was possible to evaluate the variations in relative intensities of both the components, after addition of darodipine.

The addition of darodipine led to a decrease in intensity of the e.s.r. signals of the DMPO adducts (Figure 8b and c). As shown in Table 1, such a decrease is dependent on both the kind of radical and the concentration of darodipine added to the solution (50 nM in (b) and 500 nM in (c). To quantify the variation of intensity we performed a subtraction-addition procedure (by means of the software interfaced to the e.s.r. spectrometer) which allowed us to separate the two e.s.r. components and to obtain different spectra for the two signals corresponding to the two radicals. A double integration of the two separate signals gave the relative intensities of the signals themselves. For an easier comparison of the results from the different samples, the variation in the intensities of the e.s.r. spectra was evaluated as the percentage of the decrease in signal intensity. The results of such an analysis are summarized in Table 1.

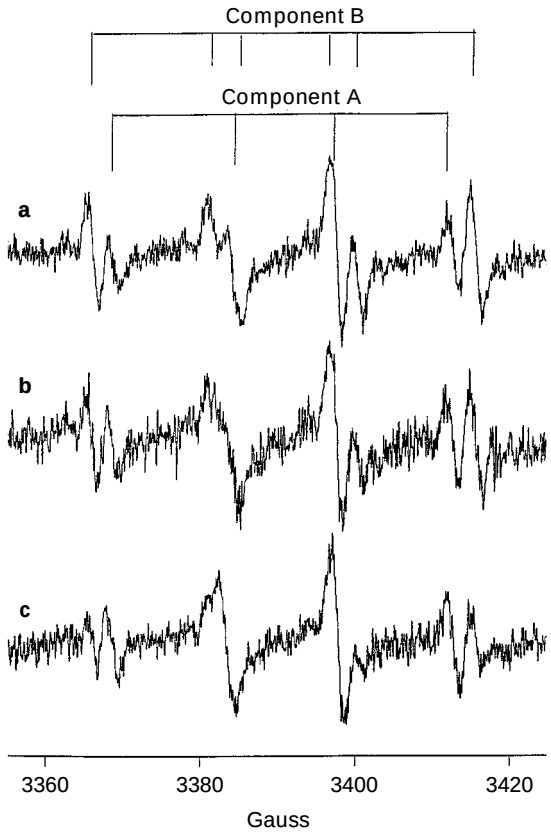

Figure 8 Representative e.s.r. spectra of DMPO radical adducts in DHF-containing solution, in the absence (a) and presence of 50 nM (b) and 500 nM (c) darodipine.

Table 1 Effect of the addition of darodipine (Daro) on signal intensity of e.s.r. spectra

Daro (nM)	*DMPO adduct*	*Intensity decrease*
50	DMPO-$COO^{\cdot -}$	37%
500	DMPO-$COO^{\cdot -}$	45%
50	DMPO-$OH^{\cdot}$	20%
500	DMPO-$OH^{\cdot}$	22%

Discussion

The present results demonstrate that darodipine exerts a concentration-dependent blockade of $I_{Ca,L}$ on rat ventricular myocytes. At a concentration which had a small effect on $I_{Ca,L}$ and which did not affect action potential configuration and contractility, darodipine protected the myocytes from the electrophysiological alterations caused by free radicals. This protective effect was demonstrated to be statistically significant in tests involving time-dependent free-radical-induced action potential prolongation, development of early afterdepolarizations and automaticity. However, darodipine did not prevent free-radical-induced depolarization of membrane potential. The protective effect of darodipine is possibly the consequence of its direct antioxidant activity.

Free radicals generated by DHF cause a variety of electrophysiological alterations in myocytes of different species (Barrington *et al*., 1988; Beresewicz & Horackova, 1991; Jabr & Cole, 1993; 1995). These data confirm and extend our previous observations in guinea-pig and rat ventricular myocytes (Cerbai *et al*., 1991; Mugelli *et al*., 1995; Guerra *et al*., 1996). The cellular electrophysiological alterations caused by DHF are, in our experience, completely prevented by free radical scavengers such as superoxide dismutase and catalase (Cerbai *et al*., 1991). Our preliminary observation (Barbieri *et al*., 1994) that darodipine, a dihydropyridine calcium antagonist, was able to prevent the free-radical-induced electrophysiological alterations in rat ventricular myocytes, prompted us to investigate further its activity and mechanism of action. First of all we wanted to exclude the possibility that the calcium antagonistic properties were not essential for this protective effect.

For this reason we characterized its calcium antagonistic properties in rat ventricular myocytes: our data clearly show that darodipine exerts selective calcium antagonistic properties by interfering with the calcium channel in a manner similar to other dihydropyridines (Cerbai *et al*., 1997a,b). We chose a concentration of darodipine which exerted a small effect on calcium current and was unable to modify the action potential configuration and contractility. Some of the ionic alterations following exposure to an oxygen radical generating solution, which in turn modify the action potential profile, are due to a change in intracellular calcium. Action potential prolongation by DHF is largely prevented by keeping intracellular calcium low by perfusing the cell with internal EGTA (Cerbai *et al*., 1991; Jabr & Cole, 1993; Mugelli *et al*., 1995). However Jabr & Cole (1995) demonstrated that exposure of guinea-pig ventricular myocytes to DHF induced a sustained depolarization which could not be prevented by intracellular EGTA, suggesting that some of the alterations caused by oxygen radicals are independent of alterations in $[Ca^{2+}]_i$. The concentration of darodipine used in these experiments excludes a major effect on intracellular calcium since it does not modify basal contractility. Furthermore, the cellular calcium overload caused by free radicals does not occur via an increase in $I_{Ca,L}$; in fact, both $I_{Ca,L}$ and DHP binding sites are reduced by free radicals generated by DHF (Guerra *et al*., 1996). Thus, it is unlikely that the protective effect of darodipine is due to its calcium antagonistic properties. It may seem surprising that cell contractility is reduced when the intracellular calcium is increased. However, in experiments in which $[Ca^{2+}]_i$ and force have been simultaneously measured (Gao *et al*., 1996), it has been clearly documented that OFR may decrease contractility even in the

presence of an increase in $[Ca^{2+}]_i$. It has been suggested that OFR may depress contractility by reducing activator Ca^{2+} availability or by decreasing Ca^{2+} sensitivity of the myofilaments (Gao *et al*., 1996). The observation that the protective effect of darodipine does not include all the alterations caused by free radicals, but instead reverses mainly the changes in action potential duration and development of early afterdepolarizations and abnormal automaticity is intriguing. One possibility is that darodipine may have selective scavenger action versus different radical species, which could cause different electrophysiological alterations. Obviously the extrapolation of the protective activity of darodipine observed in this cellular model to more integrated conditions will require further studies and, as a first step, its scavenging properties in plasma should be evaluated.

Aerobic oxidation of DHF generates large steady-state levels of superoxide anions, which have been suggested to generate additional active oxygen radicals capable of inducing lipid peroxidation (Halliwell, 1977; Goldberg & Stern, 1977). Superoxide radical forms an unstable spin adduct with the spin trap 5,5-dimethyl-1-pyrroline-n-oxide (DMPO) (Buettner & Oberley, 1978). The $O_2^{\cdot -}$-DMPO adduct decays to the $OH^{\cdot}$-DMPO adduct within the usual registration time of e.s.r. spectra. The decay process may proceed to different carbon centered radicals as a function of different chemical environments. For instance, in the presence of formiate or carboxylic acids the $OH^{\cdot}$ radical degenerates to the $COO^{\cdot -}$ radical (Finkelstein *et al*., 1980).

The DHF solution in the presence of the buffer contains formiate. Therefore, the addition of DMPO gives rise to the contemporaneous presence of the DMPO adducts with the $OH^{\cdot}$ and $COO^{\cdot -}$ radicals, as inferred by the magnetic parameters evaluated from the e.s.r. spectra: DMPO-$OH^{\cdot}$:$A_N = A_H{}^{\beta} = 14.7$ G; DMPO-$COO^{\cdot -}$:$A_N = 15.6$ G; $A_H{}^{\beta} = 18.8$ G.

Darodipine is responsible for the disappearance of a portion of radicals from the e.s.r. spectra. The antioxidant activity of this calcium antagonist is therefore connected to a radical-trapping effect. A samewhat similar mechanism of action has already been suggested for other calcium antagonists (Janero *et al*., 1988; Engineer & Sridhar, 1989; Ondrias *et al*., 1989; Goncalves *et al*., 1991). The relationship between the free radical scavenging properties of these drugs and their chemical structure has been analysed.

It is now thought that the non-substituted phenyl rings may be responsible for the antioxidant capacity of bepridil and flunarizine (Janero *et al*., 1988). The nitrophenyl group linked to hydropyridine in nifedipine may form nitroradicals, preventing lipid peroxidation (Engineer & Sridhar, 1989; Goncalves *et al*., 1991). Interestingly, the substitution of the nitrogroup at the meta-position in the phenyl ring, as in nimodipine and nitrendipine, does not affect peroxidation (Goncalves *et al*., 1991). Finally, radicals formed by nifedipine clearly belong to the class of nitrogen centered radicals, which gains stability due to trapping in phosphatidylcholine liposomes (Ondrias *et al*., 1989). The formation of these radicals gives proof to the role of radical scavenging of calcium antagonists. Darodipine has a particular structure: the benzofurazane group, linked to the dihydropyridine in *para* position does not present aromatic properties, which could favour the unpaired electron trapping by means of delocalization of π-electrons. A redox reaction may induce partial aromatic properties to benzofurazane, with consequent stabilization of the compound. This is probably the mechanism involved in the radical-trapping by darodipine.

The e.s.r. results also indicate that the radical trapping by darodipine is selective for particular kinds of radicals. When darodipine is added to the DHF solution the DMPO-$COO^{\cdot -}$ adduct decreases in intensity more than the DMPO-$OH^{\cdot}$ adduct. The effect appears to be specific since radical disappearance is dependent on the concentration of darodipine added.

In conclusion, darodipine exerts protective effects against free-radical-induced electrophysiological alterations independently of its calcium antagonistic properties; this effect is possibly due to trapping of specific radical species.

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