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Protection of mitochondrial integrity from oxidative stress by the triaminopyridine derivative flupirtine

Thomas Schlüter[a], Heinz Struy[b], Peter Schönfeld[a,*]

[a]*Institute for Biochemistry, Medical Faculty, Otto-von-Guericke-University, Leipziger Straße 44, 39120 Magdeburg, Germany*
[b]*Institute for Immunology, Medical Faculty, Otto-von-Guericke-University, Leipziger Straße 44, 39120 Magdeburg, Germany*

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Abstract The suitability of the triaminopyridine derivative flupirtine, an analgesic drug with antioxidative property [Gassen, M., Pergande, G. and Youdim, M.B.H. (1998) Biochem. Pharmacol. 56, 1323–1329], for the preservation of mitochondrial integrity from oxidative stress-induced damage was studied. Rat liver mitochondria were exposed to strong oxidative stress as generated by Fe^{2+} plus ascorbate. Peroxidation damage of membrane lipids was followed by the measurement of thiobarbituric acid reactive substances. Protein oxidation was estimated by electron spin resonance spectroscopy, after labeling of the 'peroxidized' mitochondria with 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl. We found that (i) low concentrations of flupirtine (10 μM) protect lipids and also proteins (with lesser efficiency) from attacks of reactive oxygen species; (ii) flupirtine remarkably delayed the decline of complex mitochondrial functions, such as the respiratory control or the Ca^{2+} retention capacity of mitochondria, under oxidative stress; and (iii) the ADP/ATP antiporter (ANT), a main component of the oxidative phosphorylation machinery as well as a core component of the permeability transition pore complex, seems to be a membrane protein particularly protected by flupirtine. In conclusion, the preservation of the Ca^{2+} buffer capacity of mitochondria and of the ANT activity against oxidative stress supports an anti-apoptotic application of flupirtine.

Key words: Mitochondrion; Oxidative stress; Electron spin resonance spectroscopy; Flupirtine; ADP/ATP antiporter; Permeability transition

1. Introduction

In the etiology of a wide variety of diseases (e.g. ischemia/reperfusion injury) or in the aging process the participation of reactive oxygen species (ROS) is now widely accepted [1,2]. Mitochondria are a major source of ROS production in the cell and are organelles which are particularly susceptible to oxidative stress (see [1,3,4] for recent reviews). Defective oxidative phosphorylation, e.g. such as in ADP/ATP antiporter (ANT)-deficient mice, elevates markedly the production of ROS [5]. In addition, highly energized mitochondria are also dangerous for the cell, since the reduced state of respiratory chain electron carriers supports the formation of superoxide by one-electron transfer reactions [3,6]. Consequently, 'mild uncoupling' of mitochondria decreases their ability to generate ROS [3,7]. Moreover, oxidative stress seems to damage the components of the oxidative phosphorylation machinery differentially [8,9]. Thus, it was found with senescent flies that the ANT was the only inner mitochondrial membrane protein exhibiting an age-associated increase in carbonyls [8]. Generally, oxidative stress decreases the activity of the components of oxidative phosphorylation and promotes the permeability transition (PT) of mitochondria, a process resulting in the loss of functional integrity [10,11]. Therefore, the discovery of compounds protecting mitochondria from radical attacks might be of great importance in the prevention or therapy of ROS-related diseases.

Flupirtine, a cationic triaminopyridine derivative (for chemical structure, see Fig. 1) used as a non-opioid analgesic with muscle relaxant properties [12], also acts protectively against oxidative stress as demonstrated in experiments with cells or 'functionally dead' mitochondria [13–15]. This observation stimulated us to characterize the potential ability of flupirtine to preserve the functional integrity of mitochondria upon oxidative stress. First, lipid peroxidation and protein oxidation were analyzed in order to gain insight in the antioxidative power of flupirtine on these membrane constituents. Second, the oxidative damage of membrane constituents was correlated with the decline of complex functional properties, such as the respiratory control, Ca^{2+} retention capacity or the exchange of adenine nucleotides across the inner mitochondrial membrane by the ANT. We found that flupirtine is able to protect significantly the functional integrity of mitochondria exposed to strong, short-term oxidative stress as generated by Fe^{2+} plus ascorbate.

2. Materials and methods

2.1. Chemicals

Atractyloside, 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO), 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl (MAL-6), safranin O, α-tocopherol, adenosine-5′-diphosphate (ADP), glutamate, malate, ethylene diamine tetraacetic acid (EDTA), ascorbate and $FeSO_4$ were from Sigma-Aldrich. Flupirtine ([2-amino-3-ethoxycarbonylamino-6-(4-fluoro-benzylamino)-pyridine maleate]) was a gracious gift from Asta Medica (Frankfurt, Germany). [^{14}C]ADP was purchased from Amersham. Sucrose was from Carl Roth (Karlsruhe, Germany). All other chemicals were of the highest analytical grade available.

*Corresponding author. Fax: (49)-391-67 13050.
E-mail: peter.schoenfeld@medizin.uni-magdeburg.de

Abbreviations: ANT, ADP/ATP antiporter; TBARS, thiobarbituric acid reactive substances; MAL-6, 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl; DMPO, 5,5-dimethyl-1-pyrrolidine-*N*-oxide; EDTA, ethylene diamine tetraacetic acid; ESR, electron spin resonance spectroscopy; RCR, respiratory control ratio; RLM, rat liver mitochondria; ROS, reactive oxygen species; $EC_{50\%}$, effector concentration; PT, permeability transition

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2.2. Isolation of rat liver mitochondria (RLM)

Female Wistar strain rats (150–250 g) were housed in air-conditioned rooms (22°C) under a 12-h light–dark cycle. Food (commercially available pellets) and water were given ad libitum. Mitochondria were prepared from liver essentially as in [10], but homogenization was done in ice-cold sucrose–Tris buffer (250 mM sucrose, 1 mM Tris–HCl buffer, pH 7.4) supplemented with 1 mM EDTA. Sucrose–Tris buffer applied in all other steps of the isolation procedure was EDTA-free. The protein content in the stock suspension was estimated by a modified biuret method.

2.3. Peroxidation of mitochondria and measurement of lipid peroxidation

Mitochondria (~4 mg protein/ml) suspended in 'peroxidation medium' (100 mM KCl, 10 mM Tris, pH 7.4) were subjected, in the absence and in the presence of antioxidants, to oxidative stress generated by 50 μM Fe^{2+} plus 100 μM ascorbate at 25°C [10]. Peroxidation of mitochondrial lipids was assessed by measuring the thiobarbituric acid reactive substances (TBARS) essentially as described [16]. Briefly, aliquots of the reaction mixture (100 μl) were removed after various incubation periods (15, 30, 45, 60, 75 and 120 min) and transferred to 1 ml of a 1:1 (v/v) mixture of acetic acid (2 M) and trichloroacetic acid (10% w/v in water). The denatured reaction mixture was centrifuged after 30 min in a benchtop centrifuge. To 450 μl of the supernatant, 800 μl of a thiobarbituric acid solution (0.37% (w/v) in 3.7 mM NaOH) were added and the solution was boiled for 30 min. The resulting optical density was recorded at 532 nm in a Perkin-Elmer Sigma 2 photometer.

2.4. Electron spin resonance (ESR) spectroscopy measurements

2.4.1. Antioxidative activity of flupirtine. The antioxidative capability of flupirtine was assessed indirectly by hydroxyl radical-DMPO spin trapping in the presence of various concentrations of flupirtine. Briefly, H_2O_2 (1 μl of a 30% solution in water) was rapidly added to 1 ml phosphate-buffered saline (PBS) medium supplemented with 0.1 or 1 mM DMPO, 0.1 mM flupirtine and 50 μM Fe^{2+}. An aliquot of the reaction mixture was immediately transferred to an ESR-quartz flat cell, and the DMPO/•OH spectrum recorded with a Bruker ECS 106 ESR spectrometer at 9.77 GHz. The instrumental operating parameters were: incident microwave power = 15.9 mW, modulation amplitude = 1 G, time constant = 1.28 ms, conversion time = 10.24 ms, field sweep = 100 G.

2.4.2. Protein oxidation. Mitochondria were treated with 'peroxidation medium' in the absence and presence of various concentrations of flupirtine as described above. Subsequently, in aliquots of the 'peroxidation medium', mitochondria were spun down, washed with ice-cold sucrose–Tris buffer, resuspended in the same buffer and, finally, the mitochondria (5 mg protein) were labeled with the protein-specific spin label MAL-6 at 4°C for about 18 h [17]. Afterwards, the reaction mixture was centrifuged and repeatedly washed with suspension medium to remove non-covalently bound spin label and, finally, the mitochondria were resuspended in 1 ml buffer. The instrumental operating parameters were the same as above. The W/S ratio of immobilized MAL-6 was estimated from the signal amplitudes (see inlet of Fig. 2). The W/S ratio is considered to be the measure of the immobilization of MAL-6 to 'free' (W) and 'hidden' (S, membrane-embedded) protein sulfhydryl groups [18].

2.5. Respiration and ANT activity

The oxygen consumption of control and peroxidized mitochondria in active and resting states (state 3 and state 4) was measured polarographically using a Clark-type electrode in a thermostated chamber maintained at 25°C. The 'respiration medium' was composed of 110 mM mannitol, 60 mM KCl, 60 mM Tris–HCl, 10 mM K_2HPO_4, 0.5 mM Na_2EDTA and 5 mM glutamate plus 5 mM malate as respiratory substrates (pH 7.4).

ANT activity in the samples of control or peroxidized mitochondria was measured at 0°C using the inhibitor stop technique [19]. Briefly, the forward exchange reaction was initiated by rapid addition of 0.1 ml 0.4 mM [^{14}C]ADP (specific activity 4500 dpm/nmol) to an aliquot of 0.3 ml mitochondrial suspension (~3 mg protein) and stopped after a 10 s incubation period by the addition of 0.2 ml 1 mM atractyloside. Non-specific binding of [^{14}C]ADP to mitochondria was estimated by the incubation of mitochondrial samples with 0.33 mM atractyloside prior to the addition of [^{14}C]ADP.

2.6. Ca^{2+} retention capacity

The Ca^{2+} retention capacity of energized RLM was measured by the incubation of mitochondria in EDTA-free 'respiration medium' (1 mg protein/ml) at 25°C, pH 7.4. Ca^{2+} was added as 25-nmol pulses to the incubation mixture up to the collapse of the membrane potential, recorded fluorometrically with safranin as a membrane potential probe [20].

2.7. Statistical and mathematical analysis

The statistical significance of the experimental data was determined by one-way analysis of variance followed by Dunnett's *t*-test. Differences are considered significant if P (two-sided) < 0.05 (SAS/STAT 6.12 software) [21].

3. Results

3.1. Peroxidation of lipids

RLM exposed to Fe^{2+} plus ascorbate-induced oxidative stress are peroxidized in a time-dependent manner as indicated by the formation of TBARS from membrane lipids (see Fig. 1). Even in the presence of low concentrations of flupirtine, such as 5 μM, TBARS formation slows down significantly. Flupirtine (effector concentration, $EC_{50\%} = 10 \pm 3$ μM; seven experiments) protects membrane lipids effectively from Fe^{2+}/ascorbate-induced oxidative stress comparable to the classical antioxidant α-tocopherol ($EC_{50\%} = 20 \pm 5$ μM; four experiments) does, whereas the highly water-soluble spin trap DMPO exerts no protection at this low concentrations (not shown). Since flupirtine was applied as a salt with maleate as the anionic constituent, we checked also a possible antioxidative effect of maleate on the peroxidative process. However, maleate was without any antioxidative activity, even at millimolar concentrations (data not shown). Furthermore, we found that the protection of membrane lipids from peroxidation by flupirtine is in line with its capability to diminish the efficacy of the spin trapping of the hydroxyl radical

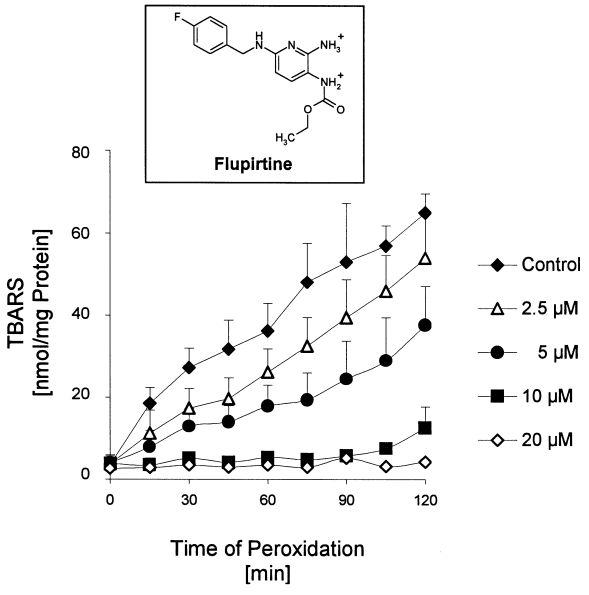

Fig. 1. Effect of flupirtine on the production of TBARS. RLM were exposed for a total incubation period of 120 min to ascorbate (100 μM)/Fe^{2+} (50 μM) in the presence of various concentrations of flupirtine. The extent of lipid peroxidation was measured as accumulation of TBARS as described in Section 2. Data are expressed as mean ± S.D. for seven independent preparations.

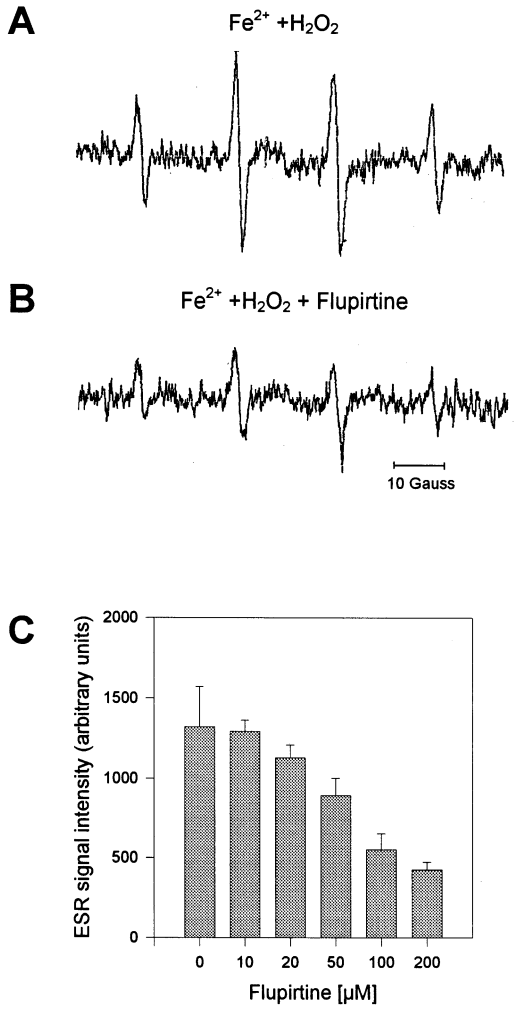

Fig. 2. Effect of flupirtine on the formation of the DMPO/•OH adduct. Hydroxyl radicals were generated by rapid mixing of 50 nmol Fe^{2+} with 32 μmol H_2O_2 in 1 ml PBS medium supplemented with 1 mM DMPO. ESR spectra of the DMPO/•OH adduct were obtained in the absence (A) or in the presence of flupirtine (100 μM; B). The dependence of the formation of DMPO/•OH adduct (100 μM DMPO) on the concentration of flupirtine is shown in (C). The results shown are the mean of four separate experiments.

by the spin trap DMPO (see Fig. 2). Thus, the concentration of the DMPO/•OH adduct obtained when DMPO was mixed with Fe^{2+} plus H_2O_2 (Fig. 2A), was clearly reduced in the presence of flupirtine (Fig. 2B,C) indicating that flupirtine competes the spin-trapping of hydroxyl radicals by DMPO.

3.2. Protein oxidation

The oxidative damage of membrane proteins was estimated by measuring the W/S ratio, which decreased in membranes exposed to oxidative stress [22,23]. For the estimation, control and 'peroxidized' mitochondria (mitochondria exposed to Fe^{2+} plus ascorbate) were labeled with the protein-specific MAL-6 and their spectra were recorded by ESR spectroscopy. Exposure of mitochondria to oxidative stress markedly reduced the W/S ratio within a short-term incubation of only 15 min (see Fig. 3A). On the contrary, when flupirtine was present no marked decline in the W/S ratio was seen. However, when RLM were exposed to longer periods of oxidative stress the W/S ratio finally declined also (see Fig. 3B). Membrane proteins are protected against oxidative stress by flupirtine with less efficiency. Thus, the W/S ratio disappeared after a 45-min exposure of mitochondria to Fe^{2+} plus ascorbate, whereas the formation of TBARS was completely suppressed after more than a 2-h exposure of mitochondria.

3.3. Respiratory control and Ca^{2+} retention capacity

The respiratory control ratio (RCR) rapidly decreased during treatment of RLM with Fe^{2+} plus ascorbate (see Fig. 4A), mainly due to an inhibition of the ADP-linked respiration (state 3). Only a 15-min exposure of RLM to oxidative stress was sufficient to half the RCR. After 45 min of oxidative stress, RLM lost their respiratory control completely. However, in the presence of flupirtine (20 μM) the decline in RCR was significantly delayed. Similar to RCR, oxidative stress reduced the Ca^{2+} retention capacity of mitochondria. The ability of 'peroxidized' RLM to accumulate Ca^{2+} from the medium is remarkably reduced after 15 min of Fe^{2+} plus ascorbate treatment. After a further 15 min of oxidative stress, mitochondria were not further able to accumulate added Ca^{2+} (see Fig. 4B). Flupirtine (20 μM) preserved the ability of RLM to accumulate Ca^{2+} from medium under short-term oxidative stress for more than 30 min. Generally, a high Ca^{2+} retention capacity indicates that mitochondria are qualified to buffer external Ca^{2+} at a low level. Exhausting the

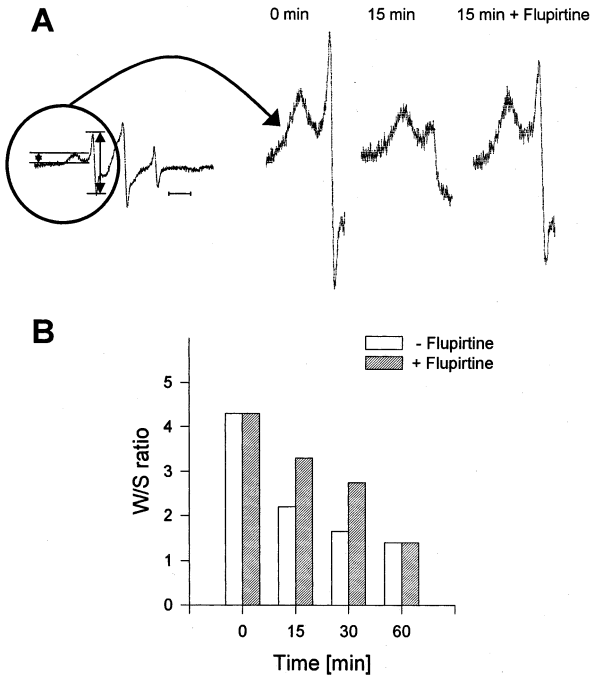

Fig. 3. Effect of flupirtine on the physical state of membrane proteins exposed to Fe^{2+}/ascorbate-generated oxidative stress. RLM were treated with Fe^{2+}/ascorbate as in Fig. 1 in the absence or presence of flupirtine (20 μM). At various incubation intervals aliquots of the reaction mixture were centrifuged. After aspiration of the supernatant, the pellet was washed with incubation medium (without Fe^{2+}, ascorbate, flupirtine). Finally, the resuspended pellet was labeled with MAL-6 as described in Section 2. A: Typical low-field ESR spectra of bound MAL-6 to control RLM and those peroxidized in the absence or in the presence of flupirtine (20 μM) are shown. B: Time course of W/S ratio during treatment of RLM with Fe^{2+}/ascorbate in the presence or absence of flupirtine (20 μM). The data shown are the mean of two independent experiments.

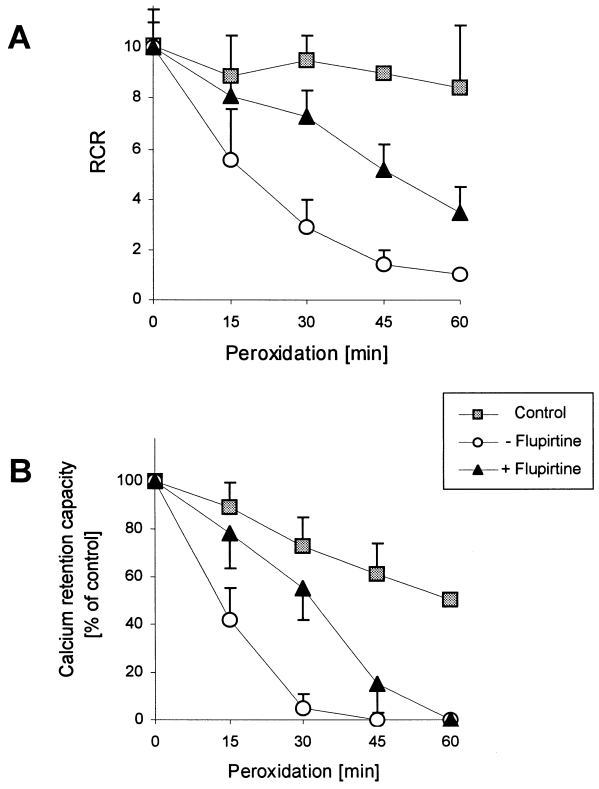

Fig. 4. Preservation of respiratory control and Ca^{2+} retention capacity. Isolation of RLM from the peroxidation mixture was carried out as in Fig. 3. A: Respiratory control was measured as in Section 2 (five separate experiments). B: Ca^{2+} retention capacity was estimated as in Section 2 (six separate experiments). The Ca^{2+} retention capacity in 'control' mitochondria was 115 ± 16 nmol Ca^{2+}/mg protein (100%). Control incubations of RLM were done in KCl–Tris medium in the absence of Fe^{2+} plus ascorbate or flupirtine.

Ca^{2+} buffer capacity is long known to trigger PT in functionally intact mitochondria (see [11] for review).

3.4. ANT activity

The ANT is a core protein of the so-called PT complex and contains oxidizable -SH groups, which are functionally essential for its activity [11,24–28]. Therefore, we studied the ability of flupirtine to preserve the ANT activity to exchange adenine nucleotides across the inner mitochondrial membrane. Treatment of RLM with Fe^{2+} plus ascorbate initiated a linear reduction in the ANT activity within 1 h (see Fig. 5). The observed decrease in [^{14}C]ADP uptake resulted from a decline in the specific ANT-based ADP uptake and, an increase in the apparent unspecific binding of [^{14}C]ADP to the pellet. When the incubation medium was supplemented with flupirtine (20 μM), the specific exchange activity was preserved to a remarkable degree for about 1 h.

4. Discussion

The results presented in this study indicate that low concentrations of the triaminopyridine derivative flupirtine protect remarkably the functional integrity of RLM exposed to strong oxidative stress. This is evidenced by preservation of the respiratory control (Fig. 4A), the Ca^{2+} retention capacity (Fig. 4B) or the activity of the ANT (Fig. 5). Especially, the ANT seems to be well protected by flupirtine against the attacks of ROS. Thus, even after 60 min of exposure of RLM to strong oxidative stress, flupirtine preserved the activity of ANT to about 50% of the control.

These observations are in line with the ability of flupirtine to inhibit powerfully the peroxidation mitochondrial membrane lipids (Fig. 1) and, with less efficiency, the oxidation of membrane proteins (see also [13]). The protection of membrane proteins against oxidative stress is clearly seen in the change in the W/S ratio in RLM exposed to oxidative stress in the absence or presence of flupirtine (Fig. 3). The oxidant-induced decrease in the W/S ratio has been attributed to an aggregation of membrane proteins, a change in protein conformation and a decrease in the amount of accessible -SH groups (see [29] for discussion).

Moreover, our results indicate further, that flupirtine probably acts as a free radical scavenger. A direct capture of ROS by flupirtine is supported by its ability to diminish the amount of the DMPO-OH adduct (Fig. 2), formed when the spin trap DMPO reacts with hydroxyl radicals (generated from Fe^{2+} plus H_2O_2). The complexation of iron ions by flupirtine as a cause of its antioxidative effect is not very likely, since, contrary to a variety of unsaturated organic compounds applied in colorimetric iron assays, we did not found that mixing of flupirtine with increasing concentrations of Fe^{2+} or Fe^{3+} changed its spectral properties (not shown). Furthermore, flupirtine protects membrane lipids against ROS attacks similar to the lipid-soluble α-tocopherol (or even more effective), while the water-soluble antioxidant DMPO was ineffective. We therefore propose that flupirtine captures ROS near the membrane, in addition to its ability to capture ROS in the bulk phase. This idea is supported by the observation that an incubation of mitochondria with increasing concentrations of flupirtine (0–50 μM) increased slightly the W/S ratio (124 ± 5% of control, three experiments), indicating that flupirtine interacts with the membrane components. However, we got no hint of an enrichment of flupirtine inside the mitochondria. A possible membrane potential-driven accumulation of the cationic flupirtine was excluded from the observation, as an addition of succinate to a mixture of antimycin A-treated RLM plus flupirtine did not change its fluorescence intensity (not shown).

Preservation of the Ca^{2+} buffer capacity and of the ANT activity makes flupirtine interesting as a tool to prevent oxi-

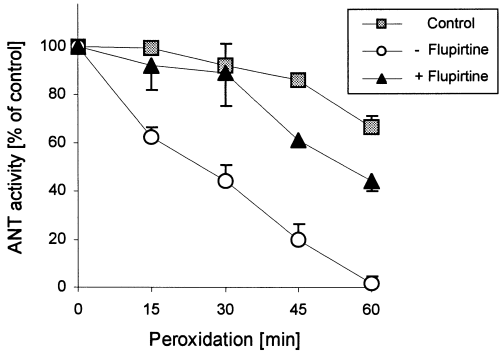

Fig. 5. Effect of flupirtine on the ANT activity exposed to oxidative stress. RLM were treated as in Fig. 3. The activity of ANT was measured as described in Section 2 (six separate experiments). The activity of ANT in 'control' mitochondria was 1.7 ± 0.3 nmol mg^{-1} $15\ s^{-1}$.

dative stress-induced apoptosis. Generally, mitochondria have been recently recognized to play a major role in the control of the apoptotic death of mammalian cells (for recent reviews, see [11,25–28]). Ca^{2+} is a classical inducer of PT. Therefore, a decreasing Ca^{2+} retention capacity reflects an increasing tendency of mitochondria to undergo PT. In addition, ANT is a central component of the so-called PT pore, a dynamic multiprotein complex formed at the contact site between the inner and the outer mitochondrial membranes [11,24–27]. It has been reported that the ANT has -SH groups that are susceptible to oxidative stress and that their oxidative modification reduces severely the effectiveness of ADP as an inhibitor of PT [11,28]. The protection of mitochondria against PT might partly explain how flupirtine counteracted apoptosis in cell cultures at oxidative stress [14,15].

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