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Effect of the triaminopyridine flupirtine on calcium uptake, membrane potential and ATP synthesis in rat heart mitochondria

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1 Flupirtine is an analgesic agent which exhibits neuronal cytoprotective activity and may have value in the treatment of conditions involving cell injury and apoptosis. Since flupirtine has no action on known receptor sites we have investigated the effect of this drug on mitochondrial membrane potential, and the changes in intramitochondrial calcium concentration in particular.

2 The findings show that flupirtine increases Ca^{2+} uptake in mitochondria *in vitro*. At clinically relevant flupirtine concentrations, corresponding to flupirtine levels *in vitro* of 0.2 to 10 nmol mg^{-1} mitochondrial protein, there was a 2 to 3 fold increase in mitochondrial calcium levels ($P<0.01$). At supra-physiological flupirtine concentrations of 20 nmol mg^{-1} mitochondrial protein and above, the mitochondrial calcium concentrations were indistinguishable from those in untreated mitochondria.

3 Mitochondrial membrane potential closely paralleled the changes in mitochondrial calcium levels showing a 20% ($P<0.01$) increase when the flupirtine concentration was raised from 0.2 nmol to 10 nmol mg^{-1} mitochondrial protein and a return to control values at 20 nmol mg^{-1} protein.

4 The increase in mitochondrial calcium uptake and membrane potential were accompanied by an increase in mitochondrial ATP synthesis (30%; $P<0.05$) and a similar percentage reduction in mitochondrial volume.

5 Calcium at 80 and 160 nmol mg^{-1} mitochondrial protein decreased ATP synthesis by 20–25% ($P<0.001$). This decrease was prevented or diminished if flupirtine at 10 nmol mg^{-1} protein was added before the addition of calcium.

6 Since intracellular levels of flupirtine in intact cells never exceeded 10 nmol mg^{-1} mitochondrial protein, these findings are supportive evidence for an *in vivo* cytoprotective action of flupirtine at the mitochondrial level.

Keywords: Ca^{2+} transport; membrane potential; mitochondria (rat heart); ATP synthesis; membrane permeability transition; swelling-contraction cycle

Introduction

Ethyl 2-amino-6-[p-fluorobenzyl)amino]-3-pyridine carbamate (flupirtine) (Figure 1) is a non-opiate analgesic with muscle relaxant activity (Moore *et al*., 1983; Schwarz *et al*., 1995) and has recently been shown to exhibit cytoprotective activity in a number of different cell models. Flupirtine protects against cell injury induced by N-methyl-D-aspartate (NMDA; Osborne *et al*., 1994) and gp120 of HIV-1 in rat cortical cells (Perovic *et al*., 1994) and against ischaemic injury in the rabbit retina (Osborne *et al*., 1996) and these effects are accompanied by a marked rise in the adenosine 5′-triphosphate (ATP) content. Flupirtine also prevents the glutamate-induced increase in intracellular Ca^{2+} content (Rupalla *et al*., 1995).

The aim of the present study in mitochondria was to investigate the mechanism by which flupirtine modulates intracellular calcium levels. The rat heart mitochondria model was selected as most suitable. For example, during ischaemia/reperfusion and depletion of high energy phosphates, the plasma membrane cannot maintain its structural integrity which is dependent on adequate concentrations of ATP (Zeman *et al*., 1990). The ensuing leakiness is followed by changes in ionic gradients in a direction opposite to that required for normal homeostasis. The concentration of intracellular calcium, which is normally four orders of magnitude lower than that in the extracellular compartment, then increases rapidly, followed by detrimental changes in the activities of phosphatases and phospholipases leading to further membrane destruction. Under these conditions, mitochondria accumulate calcium at the expense of ATP, a process which can act as an emergency function to support cellular survival. The effect of flupirtine on mitochondria may thus provide an insight into its cytoprotective action.

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Methods

Isolation of rat heart mitochondria

In general, the method of Mela and Seitz (1979) was followed. Male Wistar rats, weighing about 250 g (Hoechst Hattersheim) were anaesthetized with diethylether. The heart was removed, transferred to a medium consisting of 225 mM mannitol, 75 mM sucrose, 0.5 mM EGTA pH 7.5 at 0–4°C, cut into small pieces with scissors and washed free of blood with buffer. The tissue slurry was homogenized in an Ultraturrax (Janke & Kunkel, Staufen, Germany) for 2 s at 9500 rev min^{-1} and the suspension centrifuged for 10 min at $480 \times g$ in a Sorvall RC 5B centrifuge (4°C). The supernatant was removed and centrifuged for a further 10 min at $7710 \times g$. Mitochondria were resuspended in a EGTA-free buffer consisting of 175 mM sucrose, 90 mM mannose, 8.5 mM TES, 7 mM K_2HPO_4 at pH 7.0.

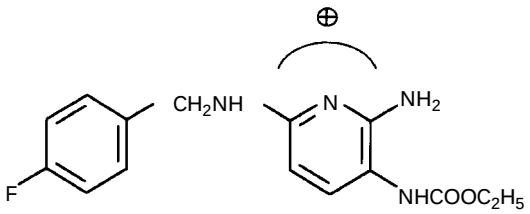

Figure 1 Structure of flupirtine.

Measurement of calcium uptake

Mitochondria (0.1 mg of protein) were suspended in 2 ml of buffer (175 mM sucrose, 90 mM mannose, 7 mM K_2HPO_4, 8.5 mM TES pH 7.0) supplemented with 1.5 mM succinate. Flupirtine was added at concentrations in the range 0.2–40 nmol mg^{-1} protein and incubated for 3 min with stirring. After the addition of INDO-1 AM (0.1 nmol mg^{-1} protein) incubation was continued for a further 1 min and the fluorescence recorded with a Perkin Elmer LS-3 spectrofluorimeter at excitation wavelength of 355 nm and emission of 405 nm. The emission was also determined at 485 nm (temp. 25°C). These measurements were repeated after addition of $CaCl_2$, 80 or 160 nmol mg^{-1} protein and the calcium content of the mitochondria calculated according to Millot *et al.* (1995), by use of the formula:

$$[Ca^{2+}]_i = K_d \times \beta \times [(R - R_{min})/(R_{max} - R)],$$

where $[Ca^{2+}]_i$ = internal calcium; K_d = dissociation constant for binding INDO-1 with Ca = 250 nM; R = value of fluorescence at 405/485 nm; R_{min} = minimal fluorescence emission 405/485 nm at zero calcium; R_{max} = maximal fluorescence emission 405/485 nm at calibration; β = ratio of fluorescence emission at 485 nm in the absence of calcium vs Ca^{2+} saturation.

Measurements of mitochondrial membrane potential

Mitochondrial membrane potential was determined with the same buffer as that described under measurements of calcium uptake and containing 1.5 mM succinate. Mitochondrial protein (0.1 mg) was stirred with the buffer for 3 min with or without flupirtine in the concentration range 0.2–40 nmol mg^{-1} protein DASPEI (3.5 nmol mg^{-1} protein; Bereiter-Hahn, 1976) was added and fluorescence recorded over a period of 3 min at 25°C at an excitation wavelength of 467 nm and emission of 555 nm. The K^+-gradient of the inner mitochondrial membrane (Åkerman & Wikström, 1976) was equivalent to 190 mV (Thürich *et al.*, 1997), calculated with the Nernst equation. The magnitude of the membrane potential was used to assess mitochondrial integrity.

Measurement of mitochondrial ATP synthesis

The experimental procedure used was the same as that described for mitoplasts and submitochondrial particles (Zimmer *et al.*, 1995a). Succinate (5 mM) was used as substrate in the same buffer as that used previously (10 mM KH_2PO_4, 5 mM $MgCl_2$, 10 mM sucrose; ADP concentration 0.5 nmol 250 μl^{-1}, 5 μg protein).

Measurement of mitochondrial ATP synthesis in the presence of calcium

Mitochondria were incubated in EGTA-free medium with or without calcium, 80 or 160 nmol mg^{-1} protein, for 2 min. Thereafter, an aliquot of mitochondrial suspension containing 5 μg of protein was added to the medium (succinate, KH_2PO_4, $MgCl_2$, sucrose, ADP, see above) for measurement of ATP synthesis.

Alternatively, mitochondria were incubated in the isolation medium for 1 min with 10 or 20 nmol mg^{-1} protein of flupirtine, and, subsequently calcium, 80 or 160 nmol mg^{-1} protein, were added. After incubation for 2 min, the aliquot, containing 5 μg of protein, was added to the medium for measurement of ATP synthesis.

Determination of mitochondrial volume changes

Mitochondrial volume changes were determined by use of measurements of light scattering as indices for a change in mitochondrial density. Mitochondrial protein (0.1 mg) was pipetted into 2 ml of the same buffer as that used for ATP synthesis at 25°C and 200 μM ADP after 2 min. Light scattering during equilibration with the buffer was recorded at 570 nm with the Perkin Elmer LS-3 spectrofluorimeter. A decrease in light-scattering is associated with swelling of the organelle.

Substances

1-[2-Amino-5-(6-carboxyindol-2yl) phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-N,N,N′,N′-tetraacetic acid pentaacetoxy methylester (INDO 1-AM) and (2-{[tris-(hydroxymethyl)-methyl]amino}ethanesulphonic acid) (TES) were obtained from Calbiochem (Bad Soden, Germany) and Chelex-100 from Bio Rad (München). 2-(Dimethyl-aminino-styryl)-1-ethylpyridiniumiodine (DASPEI) was purchased from Sigma (Deisen-hofen, Germany), succinate from Serva (Heidelberg), ADP from Boehringer (Mannheim) and ATP-monitoring reagent from Colora (Lorch). Flupirtine (Figure 1) was supplied by ASTA Medica.

All buffer salts were either p.a. or of the finest quality available. Ca^{2+} was removed by filtration through a column (250 × 25 mm) of Chelex-100 granules.

Statistical treatment of data

Multiple *t* tests were performed, with α adjustment by Bonferroni-Holm. Gauss distribution was tested according to Kolmogoroff-Smirnoff. Student's *t* test was applied.

Results

At clinically relevant flupirtine concentrations, corresponding to flupirtine levels *in vitro* of 2 to 10 nmol mg^{-1} mitochondrial protein, there was a 2 to 3 fold increase in mitochondrial calcium levels ($P < 0.01$ (Figure 2)). At 20 and 40 nmol flupirtine mg^{-1} mitochondrial protein, mitochondrial calcium was indistinguishable from that in untreated mitochondria (Table 1). At 2–10 nmol flupirtine mg^{-1} mitochondrial protein, the maximum increase in mitochondrial Ca^{2+} was reached within 30 min (Figure 3).

Mitochondrial membrane potential closely paralleled the changes in mitochondrial calcium levels showing a 20% ($P < 0.01$) increase when the flupirtine concentration was raised to 10 nmol mg^{-1} mitochondrial protein (Figure 2) and a return to control values at 20 nmol mg^{-1} protein (Table 2). A concentration of 40 nmol mg^{-1} was clearly toxic. However, significant increases were already detectable at 0.2 nmol mg^{-1} protein.

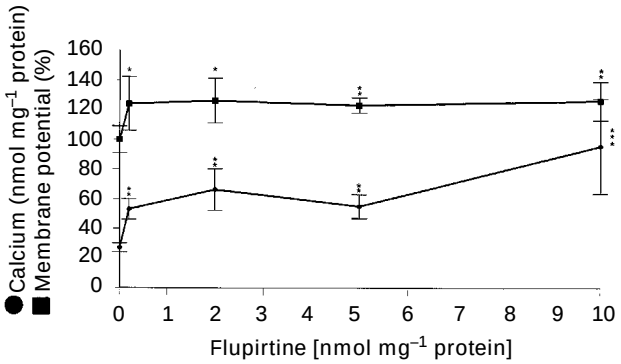

Figure 2 Determination of mitochondrial membrane potential and Ca^{2+} content, mean values are shown; $n=4-12$, and denotes the number of individual mitochondrial preparations investigated; vertical lines show s.d. $*P<0.05$, $**P<0.01$, $***P<0.001$.

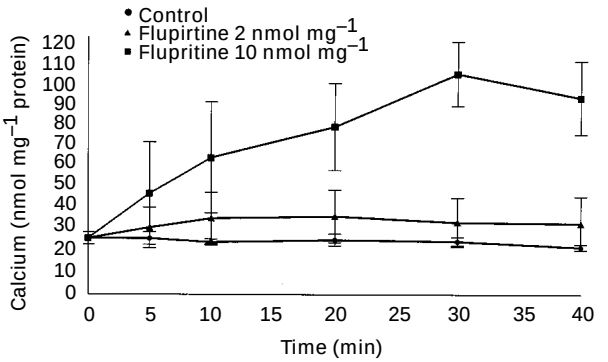

Figure 3 Time course of the increase in mitochondrial Ca^{2+} content in the presence of 2 and 10 nmol flupirtine mg^{-1} protein versus control. Vertical lines show s.e.mean.

These changes were accompanied by increases (approximately 30%, $P<0.05$) in mitochondrial ATP synthesis, which were determined at flupirtine concentrations up to 10 or 20 nmol mg^{-1} mitochondrial protein (Table 3).

Addition of calcium (80 or 160 nmol mg^{-1} protein) to mitochondria decreased ATP synthesis by about 20 or 25%. Prior addition of flupirtine at 10 nmol mg^{-1} protein preserved ATP synthesis (Table 4), which was insignificantly changed vs control at 80 nmol Ca^{2+} and 20 nmol flupirtine mg^{-1} protein. Whereas the flupirtine effect (10 nmol mg^{-1}) decreased somewhat at 160 nmol Ca^{2+} mg^{-1} protein, 20 nmol mg^{-1} proved highly effective at both concentrations of Ca^{2+} (Table 4).

Evidence for a protective effect of flupirtine on mitochondrial integrity was seen in the reduced mitochondrial swelling at the commencement of the equilibration process in buffer containing ADP (Figure 4). In the presence of flupirtine 10 nmol mg^{-1} protein. ADP produced a reduction in mitochondrial volume without initial swelling. At concentrations below or above this optimal concentration the addition of ADP caused mitochondrial swelling in the initial phase of the incubation.

Discussion

In this study we have investigated the effect of the drug flupirtine on the structure, the membrane potential, ATP synthesis and the uptake of calcium by rat heart mitochondria. If the structure of the mitochondria is preserved, one can then assume that a cytoprotective process is present. Supportive evidence for cytoprotection by flupirtine is provided by the findings of Perovic *et al.* (1994) in rat cortical cells and Osborne *et al.* (1996) in the rabbit retina.

Table 1 Calcium-uptake

Flupirtine (nmol mg^{-1} protein)	n*	*Calcium-uptake* (nmol mg^{-1} protein)	*Significance vs control* (P)
Control	12	27.1 ± 3.1	
5	5	57 ± 6.5	>0.0001
10	8	95.5 ± 32	>0.001
20	4	32 ± 10	NS
40	4	25 ± 2	NS

* n = number of individual mitochondrial preparations.

Table 2 Membrane potential in flupirtine-treated mitochondria

Flupirtine (nmol mg^{-1} protein)	n*	*Membrane potential* (% of control)	*Significance vs control* (P)
Control	6	100 ± 9	
10	8	121 ± 14	<0.01
20	4	99 ± 26	NS
40	4	69 ± 22	<0.05

* n = number of individual mitochondrial preparations.

Table 3 ATP synthesis in flupirtine-treated mitochondria

Flupirtine (nmol mg^{-1} protein	n*	*ATP synthesis* (μmol mg^{-1} protein × min)	*Significance vs control* (P)
Control	5	0.150 ± 0.018	
2	3	0.174 ± 0.028	ns
10	4	0.210 ± 0.047	<0.05
20	4	0.203 ± 0.034	<0.05

* n = number of individual mitochondrial preparations.

Table 4 ATP synthesis in flupirtine-treated mitochondria under the influence of 80 and 160 nmol mg^{-1} protein calcium

Ca^{2+} (nmol mg^{-1})	*Flupirtine* (nmol mg^{-1})	n	*ATP synthesis* (nmol mg^{-1} × min)	*Significance* (P)
Control		5	0.144 ± 0.011	
80		5	0.113 ± 0.008	<0.001 *
160		5	0.108 ± 0.007	
80	10	4	0.137 ± 0.01	<0.01 **
160	10	4	0.123 ± 0.005	<0.02 ***
80	20	4	0.134 ± 0.011	NS vs control
160	20	4	0.129 ± 0.012	NS vs control

Significance: * vs control; ** vs 80 nmol mg^{-1} Ca^{2+}; *** vs 160 nmol mg^{-1} Ca^{2+}.

Flupirtine levels in vivo

An important question is the range of concentrations over which we observed the effects on the mitochondria. This must correlate with the concentrations of the drug which are obtainable *in vivo*. The calculated *in vivo* concentration is 1–

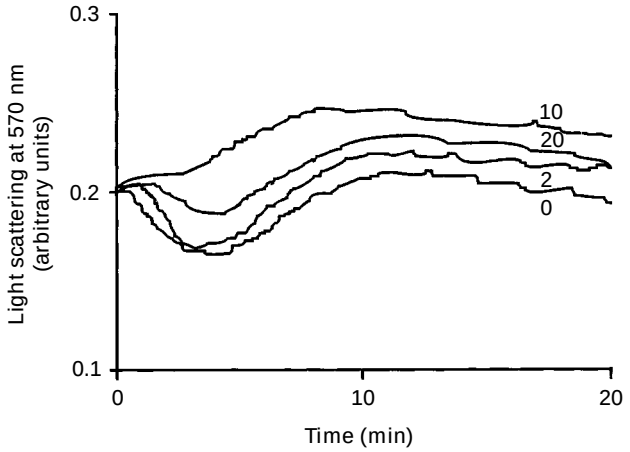

Figure 4 Effect of flupirtine on mitochondrial volume determined by use of light-scattering measurements at 570 nm. The numbers on the curves are the concentrations of flupirtine used (0.2.10 and 20 nmol mg^{-1} protein). Reaction started by ADP at time 0: representative experiment.

2.5 μg ml^{-1} at its maximum (Hlavica & Niebsch 1985). This corresponds to a plasma concentration of 6 nmol ml^{-1}. If we want to compare the *in vivo* situation with our experimental conditions, then we must carry out the following calculation: 1 g rat heart corresponds to 1 ml of volume, and if there is complete equilibrium between intra- and extracellular space it would contain about 6 nmol of flupirtine. In 1 g of rat heart there are approximately 30 mg of mitochondria (Palmer 1977; Zimmer, 1995b). On a per mg basis there would therefore be 0.2 nmol of flupirtine mg^{-1} of mitochondrial protein, assuming that *in vivo* there is complete equilibrium between intra- and extracellular space. In the cytoplasma, along with mitochondria, there are also other organelles and protein, and the binding to these sites (protein binding of flupirtine is about 80%, personal communication Dr Niebch) must be subtracted from the 0.2 nmol of flupirtine mg^{-1} of mitochondrial protein. On the other hand, an equilibrium in the living organism cannot be maintained over a long period unchanged: here, an intracellular concentration higher than 0.2 nmol mg^{-1} of mitochondrial protein would result. According to these assumptions we must add something to the calculated value of 0.2 nmol mg^{-1}.

Effects of therapeutic and toxic concentrations in vivo

Figure 2 shows that there was a significant increase in the calcium uptake ($P<0.01$) compared with the control value when about 0.2 nmol flupirtine mg^{-1} mitochondrial protein was used. This is of interest because it supports the clinical relevance of the concentrations investigated in comparison to the *in vivo* situation. On the basis of this concentration, we have investigated 100–200 times higher concentrations of flupirtine. The substance is quite readily soluble in water (1 g 100 ml^{-1}) and we can conclude therefore that no *in vivo* situation exists where an intracellular accumulation of 20–40 nmol mg^{-1} mitochondrial protein can be attained. Nevertheless, these supraphysiological concentrations have been used in order to delineate the toxic range. In the toxic range of 20–40 nmol mg^{-1} protein it was found that the intramitochondrial amounts of calcium were indistinguishable from the control value. Similarly, mitochondrial membrane potential between 20 and 30 nmol flupirtine mg^{-1} protein was in the control range and became significantly decreased to 69% of the control in the clearly toxic range of 40 nmol of flupirtine mg^{-1} protein. After a dose-dependent increase of ATP synthesis up to 10 nmol mg^{-1} protein of flupirtine, the value at 20 nmol mg^{-1} protein remained nearly stable in a range significantly above the control, with no further increase. Flupirtine 20 nmol mg^{-1} protein also decreased the effect on membrane structure seen with 10 nmol mg^{-1} protein. The only case where we observed a significant effect of 20 nmol flupirtine mg^{-1} protein was after a challenge of mitochondria with 160 nmol calcium mg^{-1} protein. The rise in ATP synthesis which we measured under these conditions was such that the difference compared with controls was insignificant.

Modulating effect of Ca^{2+} concentration in vivo

Another point of interest to us is the Ca^{2+} concentration used of 80–160 nmol mg^{-1} mitochondrial protein. Estimation of the Ca^{2+} uptake in the mitochondria was carried out with 0.1 mg mitochondrial protein 2 ml^{-1} buffer. It can therefore be calculated that 1 mg protein is contained in 20 ml buffer. Ca^{2+} 80 nmol 20 ml^{-1} buffer, i.e. per 1 mg mitochondrial protein, when extrapolated to 1 l, gives $80 \times 50 = 4$ μM Ca^{2+}. In our experiments we used 80–160 nmol Ca^{2+} mg^{-1} mitochondrial protein corresponding to 4–8 μM Ca^{2+}. These are concentrations which are clearly outside the physiological concentration range of 0.1–1 μM. Under our experimental conditions, 10–20 nmol flupirtine mg^{-1} protein was sufficient to maintain the ATP synthesis activity even in a concentration range of 80–160 nmol Ca^{2+} mg^{-1} protein ($P<0.02$). This means therefore, that the mitochondrial structure and the function were intact. A 'fluid' and intact membrane is necessary for the maintenance of ATP synthesis in the normal range. When these conditions are not met, the required membrane potential cannot be maintained at a sufficiently high level. With adequate concentrations of flupirtine (10–20 nmol mg^{-1} mitochondrial protein) the ATP synthesis was significantly increased (see Table 3). The clearly improved swelling curve in the presence of ADP was obtained at flupirtine concentrations of 10 nmol mg^{-1} mitochondrial protein. However, this does not exclude the possibility that concentrations as low as 0.2 nmol mg^{-1} protein have a significant positive effect on the Ca^{2+} uptake ($P<0.01$). Fluorometric measurements (membrane potential and Ca^{2+} uptake) are, from the point of view of sensitivity, superior to ATP synthesis measurements and measurements of the swelling (both photometric).

Mitochondrial swelling and cytoprotective action of flupirtine

Apoptosis is generally associated with mitochondrial swelling, permeability transitions and degradation (Bernardi *et al*., 1994; Zamzami *et al*., 1996). A disturbance of ATP synthesis and Ca^{2+} uptake probably precede both these changes, since these phenomena are dependent on the structural integrity of the mitochondrial membrane and the reductive state of (vicinal) -SH groups (Zimmer & Freisleben, 1988; Zimmer *et al*., 1990). Moreover, permeability alterations and oxidation of membrane sulphydryl groups result in an increase in rigidity and leakiness of the mitochondrial membrane, changes which will tend to accelerate the apoptotic cascade. The swelling of mitochondria, which occurs during equilibration with physiological buffers, is decreased by ADP (Packer, 1960). The cytoprotective action of flupirtine may be mediated, at least in part, by a potentiation of this inhibitory effect of ADP on mitochondrial swelling. Zimmer (1970) demonstrated that

ADP, when present in appropriate concentrations, can preserve sulphydryl-groups, an effect confirmed by the recent studies of Bernardi & Petronilli (1996).

The above findings of increased mitochondrial calcium uptake with flupirtine also throw light on the observations of Rupalla *et al*. (1995), who showed that flupirtine protects neurones against ischaemic damage and inhibits the increase in cytosolic Ca^{2+}. The 0.2 μM decrease in cytosolic Ca^{2+}, which was associated with a flupirtine level of approximately 0.8 μM, could be attributable to a protective action of flupirtine on the neuronal mitochondria alone. According to the observations presented here, the mitochondrial protein in the neurones investigated by Rupalla *et al*. (personal communication), was in fact sufficient to take up a considerably higher amount of Ca^{2+}, i.e. 3–4 μM.

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