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EFFECT OF TRIFLUOPERAZINE ON SKELETAL MUSCLE MITOCHONDRIAL RESPIRATION

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The effect of trifluoperazine on the respiration of porcine liver and skeletal muscle mitochondria was investigated by polarographic and spectroscopic techniques. Low concentrations of trifluoperazine (88 nmol/mg protein) inhibited both the ADP- and Ca^{2+}-stimulated oxidation of succinate, and reduced the values of the respiratory control index and the ADP/O and Ca^{2+}/O ratio. High concentrations inhibited both succinate and ascorbate plus tetramethyl-*p*-phenylenediame (TMPD) oxidations, and uncoupler (carbonyl cyanide *p*-trifluromethoxyphenylhydrazone) and Ca^{2+}-stimulated respiration. Porcine liver mitochondria were more sensitive to trifluoperazine than skeletal muscle mitochondria. Trifluoperazine inhibited the electron transport of succinate oxidation of skeletal muscle mitochondria within the cytochrome *b*-c_1 and cytochrome c_1-aa_3 segments of the respiratory chain system. 233 nmol trifluoperazine/mg protein inhibited the aerobic steady-state reduction of cytochrome c_1 by 92% with succinate as substrate, and of cytochrome *c* and cytochrome aa_3 by 50–60% with ascorbate plus TMPD as electron donors. Trifluoperazine can thus inhibit calmodulin-independent reactions particularly when used at high concentrations.

Introduction

Phenothiazine and related compounds are antipsychotic drugs widely used as probe indicators for calmodulin involvement in cellular functions [1–8], particularly in studies on calcium-dependent processes [1–5,8,9]. Recent investigations [9–13], however, have shown that these compounds are not specific inhibitors of calmodulin-dependent enzymes. For example, trifluoperazine can inhibit rat liver mitochondrial ATPase [10], ADP-stimulated oxidation of succinate and Ca^{2+} efflux [11] of rat liver mitochondria, affect lymphocyte lactate and ATP levels [12] and inhibit the noncalmodulin-dependent enzyme, protein kinase C [13]. Even though this phenothiazine compound exhibits specific calcium-dependent binding to calmodulin [1,14], thereby inhibiting its regulatory functions [1,2,4,5,15], recent reports [15–17] also suggest that trifluoperazine can bind to calcium-dependent proteins other than calmodulin.

Except for the report [10] of the inhibition of trifluoperazine of rat liver mitochondrial ATPase and ADP-stimulated oxidation of succinate, no studies on other mitochondrial respiration or electron transport have been published. This paper reports studies on the effect of trifluoperazine on the respiration of porcine mitochondria, particularly those from skeletal muscle. Trifluoperazine inhibits both Ca^{2+}- and ADP-stimulated respiration of porcine liver and skeletal muscle mitochondria oxidizing succinate in the presence of rotenone. High concentrations of trifluoperazine inhibit electron transport within the cyto-

Abbreviations FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, TMPD, tetramethyl-*p*-phenylenediamine

chrome b-c_1 and cytochrome c-aa_3 segments of the respiratory chain system of porcine skeletal muscle mitochondria.

Materials and Methods

Materials

ADP, antimycin A, fatty acid-free bovine serum albumin, phenazine methosulfate, rotenone and sodium succinate were purchased from Sigma Chemical Co.; sodium ascorbate and TMPD dihydrochloride from British Drug Houses, FCCP from Boehringer Mannheim; crystalline *Bacillus subtilis* proteinase (nagarse) from Teikoku Chemical Co., and all other reagents were of analytical grade Trifluoperazine was kindly supplied by Dr. C. van Hardeveld, Department of Chemical Pathology, Leiden, and by Smith Kline and French Laboratories Ltd., U K.

Methods

Skeletal muscle mitochondria were isolated from porcine longissimus dorsi using *B. subtilis* proteinase [18], and liver mitochondria by differential centrifugation [10] in 250 mM sucrose containing 1 0 mM EDTA (pH 7.4). The liver mitochondrial pellet ($7000 \times g$) was washed three times in 250 mM sucrose before being finally suspended in 250 mM sucrose (final concentration). Oxygen uptake was measured with a Clark oxygen electrode (Yellow Springs Oxygen Monitor (Model 53)) at 37°C The ADP-stimulated respiration for succinate oxidation was determined in a reaction medium (pH 7 20) containing 30 mM KCl, 6 mM $MgCl_2$, 75 mM sucrose, 20 mM KH_2PO_4 and 1 mM EDTA, and the Ca^{2+}-stimulated respiration in a medium (pH 7.20) containing 225 mM mannitol, 75 mM sucrose, 15 mM Tris-HCl and 5 mM P_i Difference spectra were recorded at −196°C in 1.0 mm light-path perspex cells with an Aminco-Chance dual-wavelength/split-beam (DW 1) spectrophotometer operating in the split-beam mode The steady-state reductions of the b- (562 − 575 nm) and c (554 − 540 and 550 − 540 nm)-type cytochromes and cytochrome aa_3 (605 − 635 nm) were estimated at room temperature operating in the dual-wavelength mode at appropriate wavelength pairs with an Aminco-Chance (DW 2A) dual- wavelength/ split-beam spectrophotometer using 10 mm light-path cuvettes Protein was estimated according to the method of Lowry et al [19] using bovine serum albumin as standard

Results

Inhibition of ADP- and Ca^{2+}-stimulated respiration of porcine liver and skeletal muscle mitochondria by trifluoperazine

Trifluoperazine was found to inhibit both the ADP- and Ca^{2+}-stimulated oxidation of succinate in the presence of rotenone of isolated porcine liver and skeletal muscle mitochondria (Table I). With porcine liver mitochondria the respiratory control induced by ADP was more sensitive to trifluoperazine than that induced by Ca^{2+} A loss in respiratory control was observed following the addition of 63 nmol trifluoperazine/mg protein and a complete inhibition of the ADP-stimulated respiration was observed by doubling the concentration of trifluoperazine In the case of Ca^{2+}, 63 nmol trifluoperazine/mg protein could reduce both the values of the Ca^{2+}/O ratio and the respiratory control index of a typical control experiment from 2.77 and 2 72 to 0 64 and 1 40, respectively. As in the case of ADP, a complete inhibition of the Ca^{2+}-stimulated respiration was obtained with 126 nmol trifluoperazine/mg protein Our present data thus substantiate the recent findings of the effect of phenothiazines and related compounds on rat liver mitochondria [10] but differ in two important aspects. Firstly, 4-times the concentration of trifluoperazine was required as compared with rat liver mitochondria to inhibit completely the ADP-stimulated respiration of porcine liver mitochondria. Secondly, both the ADP- and Ca^{2+}-stimulated respiration of succinate oxidation by porcine liver mitochondria was inhibited by trifluoperazine, whereas in rat liver mitochondria only the ADP-stimulated respiration was affected [10].

With skeletal muscle mitochondria higher concentrations of trifluoperazine were required to inhibit completely the ADP- and Ca^{2+}-stimulated respiration than those of liver mitochondria. Complete inhibition of respiratory control with ADP and Ca^{2+} was obtained with 264 nmol trifluoperazine/mg protein. Skeletal muscle mitochondria thus required twice the amount of trifluoperazine,

TABLE I

EFFECT OF TRIFLUOPERAZINE ON ADP AND Ca^{2+}-STIMULATED RESPIRATION BY PORCINE LIVER AND SKELETAL MUSCLE MITOCHONDRIA

The experiments were carried out polarographically at 37°C with a Clark oxygen electrode using succinate as a substrate in the presence of rotenone (2 μM) The reaction was started by addition of succinate (10 mM) followed by either ADP (200 nmol) or Ca^{2+} (300 nmol) to induce the State 3–State 4 transitions [20] in the control experiments, but ADP and Ca^{2+} were only added after trifluoperazine in the test The rates in the table are expressed in ngatom O/min per mg protein Total protein in each experiment, 5 52 mg, total volume, 2 54 ml, RCI, respiratory control index

Mitochondria	Trifluoperazine (nmol/mg protein)	Ca^{2+}				ADP			
		State 3	State 4	Ca^{2+}/O	RCI	State 3	State 4	ADP/O	RCI
Liver	0	123	45	2 77	2 72	202	47	1 60	4 30
	+63	143	102	0 64	1 40	160	160	–	1 00
Skeletal muscle	0	501	100	3 00	5 01	461	143	2 10	3 65
	+88	424	150	2 00	2 83	427	209	1 40	2 04

expressed on a protein basis, to block completely both the ADP- and Ca^{2+}-stimulated respiration of succinate oxidation as compared with porcine liver mitochondria

With both porcine liver and skeletal muscle mitochondria trifluoperazine at low concentrations could stimulate, or at high concentrations could inhibit succinate oxidation With skeletal muscle mitochondria oxidizing succinate in a medium (pH 7.20) containing 225 mM mannitol, 75 mM sucrose, 15 mM Tris-HCl and 5 mM P_i, maximal stimulation was observed with 166 ± 26 ($n = 3$) nmol trifluoperazine/mg protein, complete inhibition with 528 ± 90 ($n = 3$) nmol trifluoperazine/mg protein, and no effect at a concentration of 265 ± 7 ($n = 3$) nmol trifluoperazine/mg protein In the case of liver mitochondria, neither stimulation nor inhibition of succinate oxidation was observed with 97 nmol trifluoperazine/mg protein, and complete inhibition at 155 nmol trifluoperazine/mg protein.

Sites of inhibition of succinate oxidase system by trifluoperazine

The sites of inhibition of mitochondrial respiration by high concentrations of trifluoperazine were investigated by polarographic and spectroscopic techniques. Polarographic data (Fig 1) using succinate as a substrate showed that trifluoperazine inhibited respiration at more than one site of the electron-transport chain system of skeletal muscle mitochondria, with all the sensitive sites being localized on the oxygen side of succinic dehydrogenase (EC 1.3 99.1) Trifluoperazine had no effect on succinic dehydrogenase, since the inhibition of

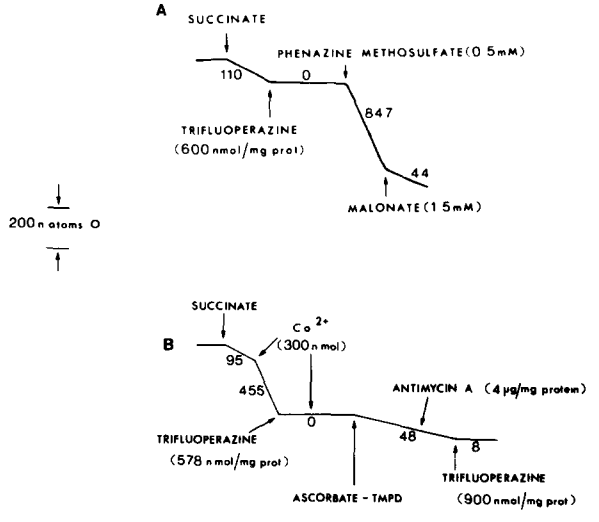

Fig 1 Polarographic experiments showing the sites of inhibition by high concentrations of trifluoperazine on the electron-transport chain system of porcine skeletal muscle mitochondria The experiments were conducted in a reaction medium (pH 7 20) containing 225 mM mannitol, 75 mM sucrose, 15 mM Tris-HCl and 5 mM P_i at 37°C Trace A represents a typical experiment showing that trifluoperazine had no affect on the succinate dehydrogenase of porcine skeletal muscle mitochondria Trace B shows that cytochrome oxidase activity, measured by the oxidation of ascorbate plus TMPD, was sensitive to high concentrations of trifluoperazine Total protein trace A, 1 30 mg, trace B, 1 12 mg Total volume, 2.60 ml Other experimental details are given in the legend to Table I

oxygen uptake due to succinate oxidation could be relieved by phenazine methosulfate, and this increase in respiration was inhibited following the addition of malonate (Fig. 1, trace A), a specific inhibitor of succinic dehydrogenase. Another site of the electron-transport chain sensitive to trifluoperazine was at the level of cytochrome oxidase (EC 1.9.3.1) (Fig. 1, trace B). The complete inhibition of succinate oxidation in the presence of Ca^{2+} by 578 nmol trifluoperazine/mg protein could be partially relieved by ascorbate plus TMPD, which donated electrons to the *c*-type cytochromes of the respiratory chain system. The cytochrome oxidase activity measured with ascorbate plus TMPD as electron donors was inhibited 80% by 578 nmol trifluoperazine/mg protein, and this activity was almost completely blocked by a total addition of 900 nmol trifluoperazine/mg protein (Fig. 1, trace B).

Difference spectra (Figs. 2 and 3) suggested that trifluoperazine inhibited succinate oxidation of skeletal muscle mitochondria by acting at two sites, one probably between the *b*- and *c*-type cytochromes and the other between the *c*-type cytochrome and cytochrome aa_3. In the presence of 880 nmol trifluoperazine/mg protein, a 15% increase in the succinate-reduced α-peak of the *b*-type cytochrome (561 nm) and a decrease of 50 and 90% in the reduced α-peak of cytochrome *c* (548 nm) and of cytochrome aa_3 (603 nm) were observed (Fig. 2, cf traces B and A). The inhibition of the reduction of these cytochromes by trifluoperazine was also supported by a substantial decrease in the reduction of the γ-peaks of these cytochromes (416 and 444 nm, respectively). Prevention of the reduction of cytochrome aa_3 by trifluoperazine was also demonstrated when

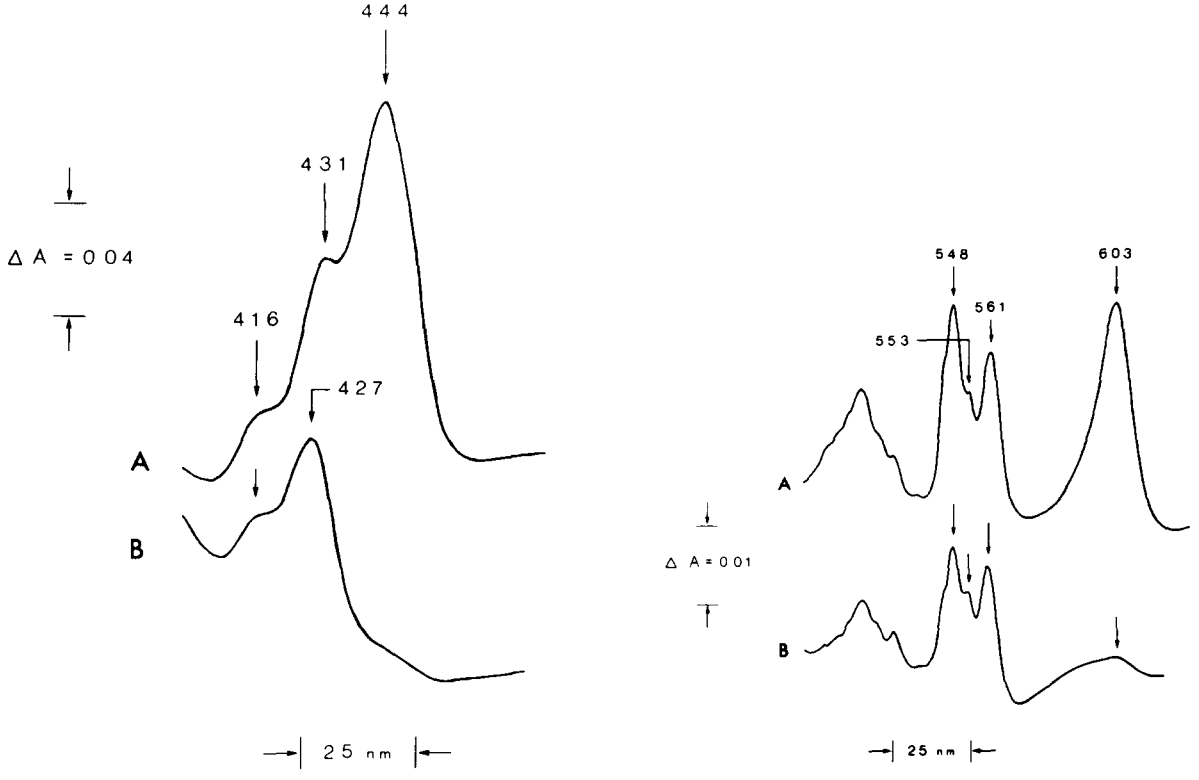

Fig 2 Difference spectra (− 196°C) showing the effect of trifluoperazine (trace B) on the succinate-reducible cytochromes in porcine skeletal muscle mitochondria (trace A) Trace A illustrates a typical control experiment showing the succinate-reduced minus oxidized difference spectrum recorded at − 196°C of the cytochromes reduced with succinate (10 mM) for 5 min at room temperature in the presence of rotenone (2 μM). The mitochondria in the reference cuvette were oxidized with air prior to freezing both cuvettes in liquid nitrogen Trace B shows the effect of 880 nmol trifluoperazine/mg protein on the succinate-reducible cytochromes in porcine skeletal muscle mitochondria Total protein in each experiment, 3 51 mg, total volume, 1 0 ml

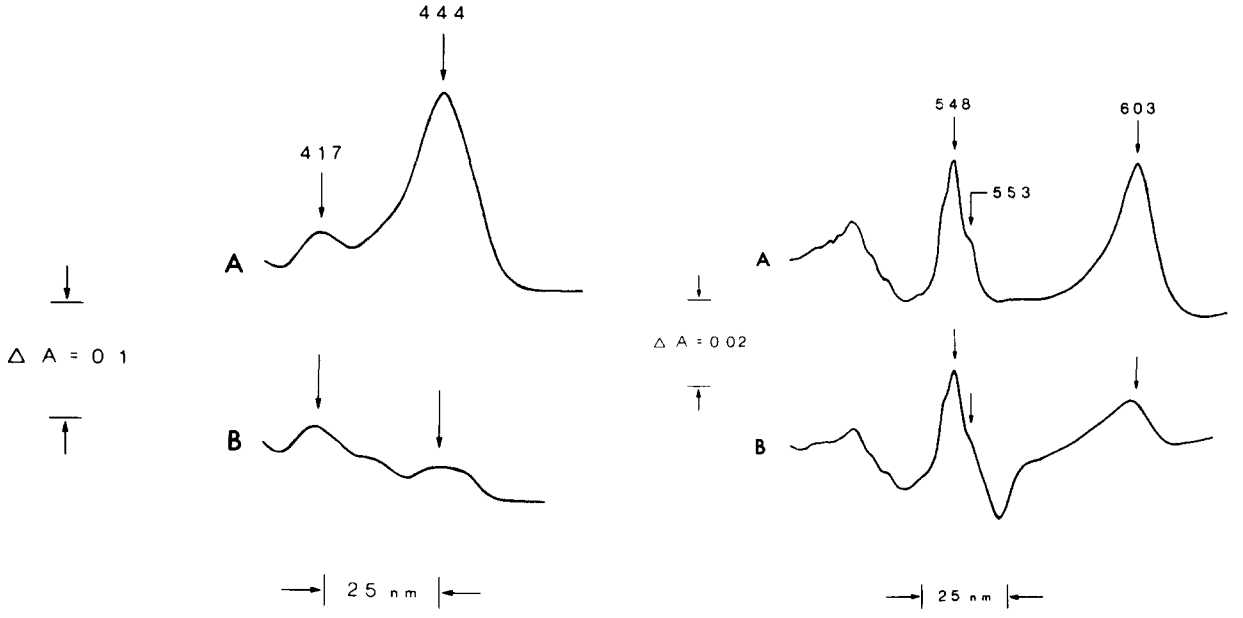

Fig 3 Difference spectra (−196°C) showing the effect of trifluoperazine (trace B) on the ascorbate plus TMPD-reducible cytochromes in porcine skeletal muscle mitochondria (trace A) Experimental conditions are similar to those described in the legend to Fig 2 except that the cytochromes were reduced with ascorbate (4 mM) plus TMPD (0 2 mM) in the presence of antimycin A (1 0 μg/mg protein) Trace A represents a typical control difference spectrum of ascorbate plus TMPD minus oxidized spectrum at −196°C, and trace B as trace A except that 880 nmol trifluoperazine/mg protein was added prior to ascorbate plus TMPD

TABLE II

EFFECT OF TRIFLUOPERAZINE ON THE AEROBIC STEADY-STATE REDUCTIONS OF CYTOCHROMES IN PORCINE SKELETAL MUSCLE MITOCHONDRIA

The reductions of cytochrome *b*, $A_{(562-575\,nm)}$, cytochrome c_1, $A_{(554-540\,nm)}$, cytochrome *c*, $A_{(550-540\,nm)}$, and cytochrome aa_3, $A_{(605-635\,nm)}$, were monitored in the dual-wavelength mode with an Aminco-Chance (DW 2A) dual-wavelength/split-beam spectrophotometer at room temperature The reaction medium (pH 7 20) contained 225 mM mannitol, 75 mM sucrose and 15 mM Tris-HCl The reduction of the cytochromes was initiated by the addition of either succinate or ascorbate plus TMPD into the magnetically stirred mitochondrial suspension in a 10 mm light-path cuvette in the control experiments, and after the addition of trifluoperazine (233 nmol/mg protein) in the test experiments Rotenone (2 μM) and antimycin A (0 5 mg/mg protein) were added to the mitochondrial suspension prior to succinate (10 mM) and ascorbate (4 mM) plus TMPD (0 2 mM), respectively Total volume, 2 56 ml, total protein, 5 37 mg –, not determined Values are expressed as %

Additions	Aerobic steady-state reductions of cytochromes			
	b (562−575 nm)	c_1 (554−540 nm)	*c* (550−540 nm)	aa_3 (605−635 nm)
Rotenone + succinate (control)	100	100	100	–
Trifluoperazine + rotenone + succinate	100	8	2	–
Antimycin A + ascorbate + TMPD (control)	–	–	100	100
Trifluoperazine + antimycin A + ascorbate + TMPD	–	–	52	40

ascorbate plus TMPD was used to donate electrons to a different site of the electron-transport chain system (Fig. 3). Under these conditions, the reduction of cytochrome aa_3 was substantially inhibited as shown by the extent of the reduced α-(603 nm) and γ- (444 nm) peaks

The aerobic steady-state reductions of these cytochromes were also modified by trifluoperazine during the oxidation of succinate and of ascorbate plus TMPD (Table II). The aerobic steady-state reduction of cytochrome c_1 was inhibited by 92% and cytochrome c by 98% with 233 nmol trifluoperazine/mg protein, which had no apparent effect on the aerobic steady-state reduction of the *b*-type cytochrome. With ascorbate plus TMPD as electron donors instead of succinate, the same concentration of trifluoperazine reduced the aerobic steady-state reduction of cytochrome c and of cytochrome aa_3 by 48 and 60%, respectively. These data suggested that the cytochrome *b*-*c* segment of the respiratory chain system was more sensitive to trifluoperazine than the cytochrome c-aa_3 segment. The effect of trifluoperazine on porcine skeletal muscle mitochondria was also reflected by the increase in the time taken before the cytochromes attained their anaerobic steady-state reductions For cytochrome c, the time taken to attain its anaerobic steady-state reduction was increased by 140% in the presence of 233 nmol trifluoperazine/mg protein, and for cytochrome aa_3 by 550%

Discussion

Our present data showing the effect of trifluoperazine on porcine liver and skeletal muscle mitochondrial function further substantiate recent evidence [9–13] that phenothiazine compounds, particularly at high concentrations, can inhibit calmodulin-independent reactions With porcine liver and skeletal muscle mitochondria, in contrast to previous findings on rat liver mitochondria [10], low concentrations of trifluoperazine inhibited both the ADP- and Ca^{2+}-respiration of succinate oxidation. Porcine liver mitochondria were found to be more sensitive to trifluoperazine than skeletal muscle mitochondria. Another interesting feature of trifluoperazine was the observed inhibition by this compound on the electron transport of succinate oxidation by skeletal muscle mitochondria, acting at two different sites of the respiratory chain system Trifluoperazine inhibited electron transport within the cytochrome b-c_1 segment of the mitochondrial respiratory chain system In this respect, this compound acted in the same fashion as antimycin A [21], 2-heptyl-4-hydroxyquinoline *N*-oxide [22], diuron [23], mucidin [24], funiculosin [25], tridemorph [26] and myxothiazol [27]. The most novel feature of trifluoperazine was its ability to block cytochrome oxidase activity by inhibiting at a site between cytochrome c_1 and cytochrome aa_3 To our knowledge, no inhibitor has yet been reported to block electron transport in the cytochrome c_1-aa_3 segment of the mitochondrial respiratory chain system

Phenothiazine compounds such as penfluridol, pimozide and trifluoperazine have recently been reported to disrupt rat liver mitochondrial energy production not associated with a phenothiazine-calmodulin interaction [10]. High concentrations of phenothiazine compounds could inhibit the uncoupler-stimulated respiration, and this was suggested to be due to gross membrane changes. The sites of inhibition were, however, not characterized [10], and could be similar to those observed in porcine skeletal muscle mitochondria.

Trifluoperazine could also inhibit the Ca^{2+} activation of phospholipase A_2 in human platelet membranes [4] and in porcine pancreas [28], with [4] or without [28] the involvement of calmodulin Inhibition of phospholipase A_2 by trifluoperazine might also be due to a nonselective hydrophobic interaction between this compound and the phospholipid substrate [17,28–30]. Indeed, low concentrations of trifluoperazine (19 μM) could overcome the Ca^{2+}-induced uncoupling of skeletal muscle mitochondria isolated from porcine malignant hyperthermia-prone pigs [18] probably by inhibiting the mitochondrial phospholipase A_2 (unpublished data) Under these conditions, the skeletal muscle mitochondria isolated from porcine malignant hyperthermia-prone pigs behaved like mitochondria isolated from normal pigs [18], by exhibiting similar values for the Ca^{2+}/O ratio and respiratory control index and accumulating a similar amount of exogenous Ca^{2+} during succinate oxidation without showing any sign of becoming uncoupled

Note added in proof (Received March 4th, 1983)

The inhibition of the Ca^{2+} activation of phospholipase A_2 in mitochondria of malignant hyperthermia-prone pigs by low concentrations of trifluoperazine is due to its inhibition on calmodulin-dependent reactions (unpublished data)

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