THE EFFECT OF 2-PHENYLINDOLONE ON PHOSPHATE TRANSPORT IN LIVER MITOCHONDRIA

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1. Introduction

Chappell and Crofts [1, 2] suggested that there was a carrier system present in liver mitochondria that facilitated the entry of phosphate or arsenate into the mitochondria. Support for this conclusion was provided by Fonyo and Bessman [3] and by Tyler [4] who showed that phosphate transport was specifically inhibited by the sulphydryl reagents *p*-hydroxymercuribenzoate and mersalyl.

In this paper we describe a new inhibitor of phosphate transport, 2-phenylindolone, and provide additional evidence for the occurrence of a specific phosphate transporter in liver mitochondria. 2-Phenylindolone was shown to strongly inhibit the mitochondrial oxidation of succinate stimulated by ADP or arsenate and the stimulation induced by calcium in the presence of phosphate. Calcium-stimulated respiration in the presence of acetate was only slightly affected by the compound. Phosphate-dependent calcium uptake and phosphate uptake were inhibited by 2-phenylindolone at the same concentrations that affected phosphate-dependent respiration. The rates of mitochondrial swelling in solutions of ammonium acetate and ammonium butyrate were not altered by the indolone, but swelling in ammonium phosphate or ammonium arsenate was strongly inhibited. It is concluded that the entry of phosphate or arsenate into rat liver mitochondria is specifically inhibited by 2-phenylindolone.

2. Methods

Tightly coupled rat liver mitochondria were prepared by the method of Chappell and Hansford [5]. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode. Mitochondrial swelling was measured as described by Fonyo and Bessman [3]. The uptake of calcium and phosphate was measured using the following methods. The suspensions obtained after incubation of the mitochondria (see legend to table 2) were decanted into 25 ml centrifuge tubes and the mitochondria were collected at 2°C by setting the speed of the MSE High Speed 18 centrifuge to maximum (18 000 rpm). After 3 min the centrifuge was turned off and the brake applied. For calcium determinations the supernatant was made up to 9 ml with water and the mitochondrial pellet was suspended in 8 ml water and sonicated for 1 min at maximum output using the large probe of the MSE 100 W sonic disintegrator. The sonicated mitochondrial suspension was made up to 9 ml with water. Both supernatant and mitochondrial suspensions were mixed with 1 ml lanthanum chloride (0.65% w/v) and the resulting precipitates were removed by centrifugation. The calcium content of the fractions was then measured using a Pye atomic absorption spectrophotometer. For phosphate determinations the mitochondrial pellet was rinsed with 5 ml ice cold Tris-sucrose buffer and the mitochondria were suspended in 4 ml water and sonicated for 1 min at maximum output from the sonic disintegrator. The sonicated suspension was then treated with 0.2 ml 60% w/v perchloric acid to precipitate the mitochondrial protein. The precipitate was removed by centrifugation and the supernatant, together with the original mitochondrial supernatant, was assayed for inorganic phosphate by the method of Fiske and Subbarow [6]. All enzyme reactions were carried out at 30°C. Protein was measured by the method of Gornall et al. [7] after solubilisation of the pellet with deoxycholate.

2-Phenylindolone was prepared by the method of Kalb and Bayer [8] and was added to the reaction medium as a solution in dimethylformamide (0.5–5.0 μl). Controls carried out with equivalent amounts of solvent had no effect on the reactions studied.

3. Results

Table 1 shows that at a concentration of 10 nmol/mg mitochondrial protein 2-phenylindolone strongly inhibited ADP-stimulated succinate oxidation, but did not affect the reaction in the absence of a phosphate acceptor, although there was some inhibition of the rate in the presence of the uncoupling agent, 2,4-dinitrophenol. The reagent markedly inhibited succinate oxidation stimulated by the addition of calcium ions when phosphate was added as the permeant anion. When the phosphate was replaced by acetate the degree of inhibition of calcium-stimulated respiration was much less marked. Succinate oxidation, stimulated by arsenate in the absence of ADP, was effectively inhibited by 2-phenylindolone. Similar results were obtained when succinate was replaced with glutamate plus malate and when p-hydroxymercuribenzoate was used instead of 2-phenylindolone.

Table 2 shows that in the absence of 2-phenylindolone the mitochondria were capable of taking up both calcium and phosphate from the suspending medium. In the presence of 2-phenylindolone the uptake of phosphate and the concomitant uptake of calcium were both severely inhibited. Since calcium uptake in the absence of phosphate was not affected by the reagent, it was thought that the primary effect of the

Table 1
The effect of 2-phenylindolone on the respiration of rat liver mitochondria.

Experiment	Additions	Rate of oxygen uptake (ngatom O/min/mg mitochondrial protein)	
		No inhibitor	With 2-Phenylindolone
1	None	20	20
	ADP + phosphate	95	24
	Calcium + phosphate	148	42
	2,4-Dinitrophenol	110	81
2	None	24	24
	Calcium + acetate	96	70
3	None	21	21
	Arsenate	63	22

The reaction mixture for experiments 1 and 2 contained 300 μmol sucrose, 50 μmol potassium chloride, 15 μmol magnesium chloride, 15 μmol sodium succinate and 75 μmol Tris-HCl pH 7.4. The reaction mixture for experiment 3 contained 10 μmol sodium succinate, 10 μmol sodium amytal, 200 μmol potassium chloride and 50 μmol Tris-HCl pH 7.4. Rat liver mitochondria (7.5 mg protein for experiments 1 and 2, and 5 mg protein for experiment 3) were added at the start of each experiment. The enzyme preparation was incubated in the reaction medium for 2 min, either in the absence of the inhibitor, or in the presence of 2-phenylindolone (10 nmol/mg mitochondrial protein) before the addition of ADP (0.5 μmol), potassium phosphate buffer, pH 7.4 (10 μmol), calcium chloride (0.75 μmol), 2,4-dinitrophenol (75 nmol), sodium acetate (10 μmol) or sodium arsenate (3.6 μmol) as indicated. The final volume was 3.0 ml.

Table 2
The effect of 2-phenylindolone on the uptake of calcium and phosphate by rat liver mitochondria.

Experiment	Conditions	μmol calcium or phosphate	
		Supernatant	Mitochondria
1	Calcium	0.55	0.70
	Calcium + indolone	1.20	0.10
2	Phosphate	0.02	0.45
	Phosphate + indolone	0.35	0.10

The reaction medium for experiment 1 contained 516 μmol sucrose, 129 μmol Tris-HCl buffer pH 7.4, 84 μmol potassium chloride, 30 μmol sodium succinate, 30 μmol magnesium chloride, 25 μmol potassium phosphate buffer pH 7.4 and 13.5 mg rat liver mitochondrial protein. The mitochondria were incubated in the reaction medium for 2 min, either in the absence or in the presence of 2-phenylindolone (10 nmol/mg mitochondrial protein), before the addition of 1.25 μmol calcium chloride to give a final volume of 5 ml. 1 min after addition of the calcium the mitochondria were collected by centrifugation at 2°C and the supernatant and mitochondrial pellet fractions were assayed as described in the Methods section. The reaction medium for experiment 2 contained 300 μmol sucrose, 75 μmol Tris-HCl buffer pH 7.4, 50 μmol potassium chloride, 15 μmol sodium succinate, 15 μmol magnesium chloride, 0.5 μmol potassium phosphate buffer pH 7.4 and 10 mg rat liver mitochondrial protein. The mitochondria were then incubated as described for experiment 1 and the supernatant and mitochondrial pellet fractions were assayed for phosphate after collection of the mitochondria by centrifugation. The final volume of the reaction mixture was 3 ml.

Table 3
The effect of 2-phenylindolone on mitochondrial swelling in solutions of ammonium salts.

Suspending medium	Rate of swelling (O.D. units/min)	
	No inhibitor	With 2-phenylindolone
Ammonium phosphate (0.1 M)	0.52	0.25
Ammonium acetate (0.15 M)	0.95	0.92
Ammonium arsenate (0.1 M)	0.60	0.30
Ammonium butyrate (0.15 M)	0.50	0.51

Rat liver mitochondria (2.5–7.5 mg mitochondrial protein) were added to a solution containing the concentrations of the ammonium salts (pH 7.4) shown in the table. Where indicated, 2-phenylindolone (10 nmol/mg mitochondrial protein) was added to the suspending medium prior to the addition of the mitochondria. The final volume of the reaction mixture was 3 ml. The rate of swelling is shown as the rate of decrease in optical density units/min, of the optical density recorded at 520 nm, 15 sec after the addition of the mitochondria.

indolone was on the phosphate transport system. To test this hypothesis further the effect of 2-phenylindolone on mitochondrial swelling in solutions of ammonium salts was examined. Chappell and Haarhoff [9] have shown that the swelling of mitochondria in ammonium salt solutions was due to penetration into the mitochondria of the anion present. Table 3 shows that the degree of mitochondrial swelling in ammonium phosphate or ammonium arsenate was strongly inhibited by 2-phenylindolone, whereas swelling in ammonium acetate or ammonium butyrate was unaffected by the inhibitor. Similar results were obtained when *p*-hydroxymercuribenzoate was used instead of 2-phenylindolone.

4. Discussion

Reactions such as succinate oxidation in the absence of a phosphate acceptor, acetate-dependant calcium-stimulated respiration, calcium uptake in the absence of phosphate and mitochondrial swelling in ammonium acetate or ammonium butyrate were not affected by 2-phenylindolone. Phosphate-dependant ADP-stimulated succinate oxidation, phosphate-dependant calcium-stimulated respiration and arsenate-stimulated oxidation of succinate were inhibited by 2-phenylindolone. The compound also inhibited calcium uptake from a phosphate containing medium, phosphate uptake and the swelling that accompanies ion uptake in ammonium phosphate or ammonium arsenate. All of the reactions sensitive to 2-phenylindolone are dependant on the transport of arsenate or phosphate from the suspending medium into the mitochondria. The reactions insensitive to the reagent do not require the transport of phosphate or arsenate, but proceed under the influence of other anions or by some alternative mechanism. The results therefore are consistent with the hypothesis that the observed effects of 2-phenylindolone on mitochondrial respiration and swelling are due to a specific inhibition of phosphate transport. The results also provide additional evidence, albeit indirect, for the proposed existance of a phosphate transporter that is also capable of transporting arsenate [1].

During the course of the present study a similar pattern of inhibitions to 2-phenylindolone was observed with the mercurial *P*-hydroxymercuribenzoate (see also [3]). The effects of mersalyl [4], another mercurial compound, also resemble those of the indolone. Particularly striking is the fact that all three compounds exhibit near maximal inhibition of mitochondrial reactions involving phosphate transport at a concentration of between 10 and 12 nmol inhibitor/mg mitochondrial protein. It would, however, be an overestimate to conclude that the concentration of the phosphate transporting catalyst is also 10 nmol/mg mitochondrial protein, since all three inhibitors are known to have effects on other mitochondrial enzyme systems. For example, mersalyl [4] and *p*-hydroxymercuribenzoate [10] have been shown to interact with the mitochondrial NADH dehydrogenase and 2-phenylindolone to inhibit transhydrogenase reactions catalysed by submitochondrial particles [11].

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