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POSSIBLE MECHANISM OF ACTION OF PERHEXILINE MALEATE ON HEART MITOCHONDRIA

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Abstract—Oxidative phosphorylation and oxidation of NAD^+-linked substrates by rat heart mitochondria were depressed by 6.75×10^{-5} M perhexiline maleate (PM), while the succinate oxidation was increased to 320 per cent activity. The drug had no effect on mitochondrial succinic and malate dehydrogenase, NADH-ferricyanide reductase and NADH-CoQ reductase (340 nm); therefore, NADH-oxidase, mitochondrial electron-transporting particles (EP_1), NADH-CoQ reductase (550 nm) and aspartate aminotransferase were inhibited. It is suggested that PM would act, preventing the reoxidation of $NADH^+ + H^+$ through the respiratory chain.

Perhexiline maleate [2-(2,2-dicyclohexylethyl)-piperidine] has been investigated to determine the absorption, excretion and metabolism in rats, dogs and humans [1–4]. However, the mechanism by which it might work is largely unexplored.

In preceding papers, a mitochondrial mechanism of action for the anti-arrhythmic drugs [5–7] was proposed. The purpose of the experiments reported below was to obtain more detailed information on the effect of perhexiline maleate (PM) on heart mitochondrial oxygen uptake and oxidative phosphorylation and to study the alterations of enzymatic systems involved with electron transport.

MATERIALS AND METHODS

Isolation of heart mitochondria. Rat heart mitochondria were isolated according to the method described for brain mitochondria by Voss *et al.* [8], using a mannitol-sucrose medium.

Methods of assay. The polarographic determinations of oxidative phosphorylation were made with an oxygen electrode as described by Voss *et al.* [9]. The P/O ratios were calculated as ADP/O ratios according to Chance and Williams [10]. Oxygen uptake was determined by Warburg respirometry. Protein was determined by the method of Lowry *et al.* [11].

Particles with NADH-oxidase activity. Submitochondrial particles with NADH-oxidase activity [12] were prepared by adopting the procedure described by Kielley and Kielley [13] for preparation of ATPase. The activity of NADH-oxidase was measured with an oxygen electrode using the following medium: phosphate buffer, 0.05 M, pH 7.5, and NADH, 3.3×10^{-4} M, in a total volume of 2.4 ml. The activity of NADH-ferricyanide reductase was measured spectrophotometrically at 420 nm (using Beckman DB spectrophotometer) with the following reaction mixture: phosphate buffer, 0.03 M, pH 7.5; $K_3Fe(CN)_6$, 1.0 mM; KCN, 1.6 mM; NADH, 0.3 mM; NADH-oxidase (0.3 mg protein); and PM, 6.75×10^{-5} M. The final reaction volume was 3.0 ml.

Heart mitochondrial electron-transporting particles (inner membrane). Beef heart mitochondria were prepared by the method of Crane *et al.* [14]. Isolation of EP_1 particles were made by the method of Kopaczyk [15] and the NADH-oxidase activity was measured polarographically using the following reaction medium [8]: mannitol, 0.25 M; Tris, 0.01 M; EDTA, 0.2 mM; KCl, 0.01 M, and inorganic phosphate, 0.1 M (final pH 7.4); EP_1 particles (2.0 mg protein); NADH, 0.3 mM; and PM, 6.75×10^{-5} M. The total volume was 2.4 ml.

NADH-CoQ reductase. Beef heart mitochondria were isolated by the method of Crane *et al.* [14]. The enzyme was prepared by the method described by Sanadi *et al.* [16]. Oxidation of NADH by CoQ was assayed spectrophotometrically (using an Amino–Chance dual wavelength/split beam recording spectrophotometer) at 340 nm and the reduction of cytochrome *c* by $CoQH_2$ at 550 nm, using the following system: Tris–sulfate buffer, 0.05 M, pH 8.0; NADH, 10^{-4} M; cytochrome *c*, 6.0×10^{-5} M; and alcoholic supernatant with NADH-CoQ reductase activity (1.20 mg protein). The total volume was 3.0 ml. To test the action of the drug, 6.75×10^{-5} M perhexiline maleate was added to the sample cuvette.

Succinic dehydrogenase. Beef heart mitochondria were isolated by the method of Crane *et al.* [14]. The enzyme was prepared by the method of Singer *et al.* [17] and assayed spectrophotometrically at 455 nm with a Varian 635D spectrophotometer according to Slater and Bonner [18]. The following system was used: phosphate buffer, 0.25 M, pH 7.6; EDTA, 30 mM; KCN, 0.1 M; succinate, 0.5 M; bovine serum albumin, 3% solution; $K_3Fe(CN)_6$, 0.1 M; enzyme (0.8 mg protein); and PM, 6.75×10^{-5} M. The final volume was 3.0 ml.

Malate dehydrogenase. Malate dehydrogenase was isolated from beef heart mitochondria [14], purified according to England and Siegel [19], and the activity was measured at 340 nm with a Varian 635D spectrophotometer, using the following system: Tris–acetate,

pH 8.0, 0.05 M, in acetate; oxalacetate, 10^{-4} M; NADH, 10^{-4} M; malate dehydrogenase, 1000 units (1 unit = absorbance change of 0.001/min); and PM, 6.75×10^{-5} M. The total volume was 3.0 ml.

Aspartate aminotransferase of human serum. The activity was measured according to Schwartz [20]. Malate dehydrogenase used was prepared according to Englard and Siegel [19]. Absorption at 340 nm was measured with a Varian 635D spectrophotometer using the following mixture: NADH, 10^{-4} M; aspartate, 4×10^{-2} M; human serum to give an absorbance change of 0.03/min; malate dehydrogenase, 1000 units (1 unit = absorbance change of 0.001/min); phosphate buffer, 0.067 M, pH 7.4; α-ketoglutarate, 7.0×10^{-3} M; and PM, 6.75×10^{-5} M. The total volume was 3.0 ml.

Determination of oxalacetate levels. Oxalacetate concentration was determined using malate dehydrogenase purified by the method of Englard and Siegel [19]. Mitochondrial suspension (5.0 mg protein) in mannitol reaction medium [8] and 4×10^{-2} M succinate was incubated at 37° for 60 min. After incubation the material was deproteinized with 2 vol. of 10% trichloroacetic acid (TCA) and centrifuged at 10,000 *g* for 10 min. The neutralized supernatant (pH 7.4 to 8.0) was used to determine the oxalacetate concentration and the enzyme activity at 340 nm was measured with a Varian 635D spectrophotometer until completion of the reaction.

RESULTS

Effect of perhexiline maleate on oxygen uptake. It has been found (Table 1) that increasing amounts of PM inhibited the oxygen uptake when glutamate, α-ketoglutarate and malate plus pyruvate were the substrates. However, when succinate was used, a significant increase in the oxygen uptake was observed. The effect of PM on the oxygen uptake of rat heart mitochondria was measured by Warburg respirometry. The data represent the average of five experiments.

Effect of perhexiline maleate on oxidative phosphorylation. The results presented in Table 2 show the effect of PM on substrate respiration, states III and IV of respiration, and ADP/O ratio.

Effect of perhexiline maleate on enzymatic activities. Since the oxidation of NAD^+-linked substrates was inhibited while that of succinate was increased, PM was tested on several enzymatic preparations related to the electron transport chain. The purpose of these studies is to locate the site of interaction of PM within the electron transport chain. The results are shown in Table 3.

Table 1. Effect of perhexiline maleate (PM) on the oxygen uptake of rat heart mitochondria*

Perhexiline maleate (M)	Activity %			
	α-Ketoglutarate	Glutamate	Malate + pyruvate	Succinate
Control	100.0	100.0	100.0	100.0
4.50×10^{-6}	88.0	70.0	100.0	116.0
2.25×10^{-5}	56.0	42.0	95.0	146.0
4.50×10^{-5}	10.0	9.0	69.0	186.0
6.75×10^{-5}	0.0	0.0	14.0	320.0

* System: reaction medium [8] (mannitol, 0.25 M; Tris, 0.01 M; EDTA, 0.2 mM; KCl, 0.01 M; and inorganic phosphate, 0.1 M, final pH 7.4); mitochondrial suspension (5.0 mg protein); 100 μmoles substrate; and 0.15 ml KOH. The experiments were carried out for 60 min. Total volume was 2.85 ml. Temperature: 37°.

Table 2. Effect of perhexiline maleate on oxidative phosphorylation by rat heart mitochondria*

Substrate	PM (M)	Substrate respiration	State III	State IV	RC	ADP/O
α-Ketoglutarate	0	0.28	1.05	0.28	3.75	3.03
	2.25×10^{-5}	0.48	1.96	0.47	4.20	2.42
	4.40×10^{-5}	0.22	0.79	0.17	4.70	3.03
	6.75×10^{-5}	0.14	0.00	0.00	1.00	0
Glutamate	0	0.36	1.21	0.36	3.37	2.98
	2.25×10^{-5}	0.36	0.98	0.21	4.67	2.98
	4.40×10^{-5}	0.21	0.42	0.24	1.75	2.78
	6.75×10^{-5}	0.11	0.00	0.00	1.00	0
Succinate	0	1.19	2.31	1.10	2.10	2.00
	2.25×10^{-5}	1.33	2.31	1.26	1.83	1.63
	4.40×10^{-5}	1.19	1.82	1.26	1.44	1.47
	6.75×10^{-5}	1.26	1.26	1.26	1.00	0

* System: reaction medium [8]; mitochondrial suspension (5.0 mg protein), 10 μmoles substrate and 235 μmoles ADP. Final volume was 2.4 ml. Data are μM O_2/sec.

Table 3. Effect of perhexiline maleate on enzymatic activities*

Activity measured	Inhibition (%)
0700NADH-oxidase	90
NADH-ferricyanide reductase	18
Elementary particles, EP_1 (NADH-oxidase)	49
NADH-CoQ reductase (340 nm)	20
NADH-CoQ reductase (550 nm)	82
Succinic dehydrogenase	0
Malate dehydrohenase	0
Aspartate aminotransferase	29

* Assays are described in Methods. PM concentration was 6.75×10^{-5} M.

Determination of oxalacetate levels. The effect of perhexiline maleate on the mitochondrial oxalacetate level was determined using the following system: neutralized supernatant from mitochondrial suspension (5.0 mg protein); Tris–acetate buffer, 0.05 M in acetate, pH 8.0; NADH, 10^{-4} M; and malate dehydrogenase, 200 units (1 unit = absorbance change of 0.001/min). The mitochondrial suspension incubated with succinate gave a concentration of oxalacetate equal to 20.0×10^{-5} M; in the presence of PM (6.75×10^{-5} M) the level of oxalacetate decreased to 2.10×10^{-5} M. An experimental control was performed without succinate, giving an undetected level of oxalacetate concentration.

DISCUSSION

In the present report, using isolated rat heart mitochondria, it has been found that increasing amounts of perhexiline maleate inhibit progressively the oxidation of α-ketoglutarate, glutamate and malate coupled with pyruvate (Table 1). Table 2 shows that substrate respiration, and states III and IV of respiration were depressed by PM when α-ketoglutarate and glutamate were the substrates, which indicates that oxidative phosphorylation or electron transport chain or both are inhibited. When succinate was used as substrate, it could be observed that substrate respiration and state IV of respiration were not significantly altered, but state III of respiration and ADP/O ratio were depressed by PM. These results suggest that the effects of PM could be by inhibition of oxidative phosphorylation and of electron transport chain on the complex I (NADH-CoQ reductase). Similar findings were obtained for propranolol [5] and iproveratril [6].

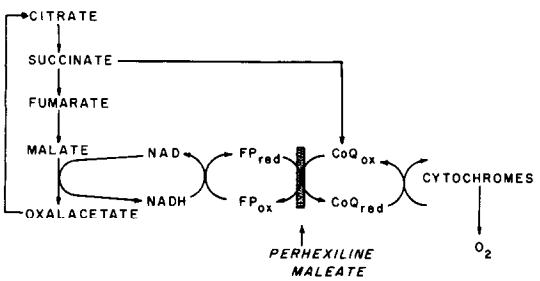

Fig. 1. Schematic representation for the possible mechanism of action of perhexiline maleate.

The evidence that perhexiline maleate has a selective action on NADH-oxidase activity is shown in Table 3. The inhibition of NADH-oxidase was reversed by methylene blue. Perhexiline maleate at a concentration of 6.75×10^{-5} M inhibited NADH-oxidase by 90 per cent, and NADH-CoQ reductase activity by 82 per cent (550 nm), while NADH-ferricyanide reductase and NADH-CoQ reductase (340 nm) were inhibited only 18 and 20 per cent respectively. This indicates that, like rotenone [21–23], propranolol [5] and iproveratril [6], the main site of action of perhexiline maleate is probably on the oxygen side of flavoprotein (Complex I).

Since perhexiline maleate did not affect the activity of succinic and malate dehydrogenase (Table 3), but enhances to 320 per cent the oxidation of succinate (Table 1), possibly preventing the reoxidation of $NADH^+ + H^+$ through the respiratory chain, it is suggested that the increase on the oxidation of succinate could be by a feedback mechanism that would decrease oxalacetate levels. Figure 1 illustrates a schematic representation for the possible mechanism of action of perhexiline maleate.

It is known that oxalacetate is probably the most potent inhibitor of succinic dehydrogenase [24, 25]. The reaction, malate → oxalacetate, is dependent on the $NADH/NAD^+$ ratio; whenever restrictions are imposed on electron transport in the respiratory chain, NADH produced by the Krebs cycle itself will not be reoxidized by NADH dehydrogenase, but will accumulate and shift the equilibrium reaction catalyzed by malate dehydrogenase in favor of malate production at the expense of oxalacetate. In this way, perhexiline maleate would decrease the oxalacetate level in favor of malate accumulation. The reaction of transamination possibly does not play an important role in the disappearance of oxalacetate, since aspartate aminotransferase activity was 29 per cent inhibited (Table 3).

Thus, perhexiline maleate seems to be a compound of considerable interest for understanding the nature of the relationship of the components in the $NADH^+ + H^+$ dehydrogenase sequence of the respiratory chain.

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REFERENCES

1. G. A. Leeson, J. F. Lang, A. V. Zeiger, W. J. Hudak and G. J. Wright, *Pharmacologist* **11,** 280 (1969).
2. G. A. Leeson, A. V. Zieger, J. F. Lang and G. J. Wright, *Fedn Proc.* **28,** 798 (1969).
3. G. J. Wright, in *Symp. on Analyses of Drugs in Biological Fluids*, p. 21. Washington. D.C. (1970).
4. G. J. Wright, A. V. Zeiger, G. A. Leeson and J. F. Lang, *Post-grad. med. J.*, (Apr. suppl.), 8 (1973).
5. A. Sakurad, D. O. Voss, D. Brandão and A. P. Campello, *Biochem. Pharmac.* **21,** 535 (1972).
6. O. Silveira and A. P. Campello, *Res. Commun. Chem. Path. Pharmac.* **10,** 149 (1975).
7. A. Sakurada, O. Silveira, M. L. Wambier, D. Brandão and A. P. Campello, *Res. Commun. Chem. Path. Pharmac.* **11,** 89 (1975).
8. D. O. Voss, A. P. Campello and M. Bacila, *Biochem. biophys. Res. Commun.* **4,** 48 (1961).
9. D. O. Voss, J. C. Cowles and M. Bacila, *Analyt. Biochem.* **6,** 211 (1963).
10. B. Chance and G. R. Williams, *Nature, Lond.* **175,** 1120 (1955).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
12. L. Ernster, in *Symp. on Biological Structure and Function* (Eds. T. Goodwin and O. Lindberg), Vol. 2, p. 139. Academic Press, New York (1961).
13. W. W. Kielley and R. K. Kielley, *J. biol. Chem.* **200,** 213 (1953).
14. F. L. Crane, J. L. Glenn and D. E. Green, *Biochim. biophys. Acta* **22,** 475 (1956).
15. K. C. Kopaczyk, in *Methods in Enzymology* (Eds. R. W. Estabrook and M. Pullman), Vol. 10, p. 225. Academic Press, New York (1967).
16. D. R. Sanady, R. L. Pharo and L. A. Sordahl, in *Methods in Enzymology* (Eds. R. W. Estabrook and M. Pullman), Vol. 10, p. 297. Academic Press, New York (1967).
17. T. P. Singer, E. B. Kearney and P. Bearnath, *J. biol. Chem.* **223,** 599 (1956).
18. E. C. Slater and W. D. Bonner, *Biochem. J.* **52,** 185 (1952).
19. S. Englard and L. Siegel, *Biochim. biophys. Acta* **54,** 67 (1961).
20. M. K. Schwartz, in *Methods in Enzymology* (Eds. R. W. Estabrook and M. Pullman), Vol. 17-b, p. 866. Academic Press, New York (1971).
21. P. E. Lindahk and K. E. Oberg, *Expl Cell Res.* **23,** 228 (1961).
22. K. E. Oberg, *Expl Cell Res.* **24,** 163 (1961).
23. L. Ernster, G. Dallner and G. Azzone, *J. biol. Chem.* **238,** 1124 (1963).
24. L. Massart, *Hoppe-Seyler's Z. physiol. Chem.* **250,** 190 (1939).
25. A. B. Pardee and V. R. Potter, *J. biol. Chem.* **176,** 1085 (1948).