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EFFECTS OF PROPRANOLOL ON HEART MUSCLE MITOCHONDRIA

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Abstract—The effects of propranolol on oxidative phosphorylation and oxidation of succinate, α-ketoglutarate, glutamate and L-malate coupled with pyruvate by rat heart mitochondria were studied. Oxidative phosphorylation and oxidation of NAD^+-linked substrates by heart mitochondria were depressed by $1{\cdot}44 \times 10^{-3}$ M propranolol. The activity of NADH-oxidase, NADH-cytochrome *c* reductase and heart mitochondrial transporting particles (inner membrane) were depressed by propranolol. Dibutyryl cyclic AMP (DBc AMP) does not overcome the inhibition caused by propranolol. It is hypothesized that propranol acts at the NAD^+-oxidase segment of the chain, its site of action being localized between NAD^+ and flavoprotein.

CURRENT concepts in adrenergic pharmacology propose the presence of two types of receptors at the adrenergic neuro-effector site, designated as alpha and beta types, according to Ahlquist.[1] Murad *et al.*[2] have reported that epinephrine stimulates the synthesis of cyclic 3′,5′-adenosine monophosphate (3′,5′-AMP) in heart muscle by a direct action on adenyl cyclase, and that this stimulatory effect is prevented by beta-adrenergic blockade. Similar findings have been reported in other tissues.[3] In the same way, the effects of other catecholamines on the particulate enzyme adenyl cyclase and the ability of various beta-adrenergic blocking agents to inhibit these effects have been thoroughly examined by Robison *et al.*[4] There have been numerous studies dealing with the effects of beta-blocking agents on the metabolic responses to catecholamines. However, it appears that less attention has been devoted to metabolic effects of the beta blockers *per se*. Investigations of these drugs on lipid and carbohydrate metabolism have already been considered.[5–8] The papers of Sobel *et al.*[9] and Wikström[10] show the effect of some adrenergic blocking agents on the normal properties of isolated mitochondria.

At the present time it is difficult to say whether the primary effect of catecholamines, and consequently, of beta-adrenergic blocking agents, is on cyclic 3′,5′-AMP formation or on the mechanisms influencing contraction, or whether these are concomitant phenomena with no immediate causal relationship. This study is concerned with the effects of propranolol on mitochondrial respiratory control and oxidative phosphorylation from normal rat hearts, besides the action of the drug on several enzymatic systems envolved with electron transport, as a contribution to elucidate the toxicological effects of the beta-adrenergic blockers.

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MATERIAL AND METHODS

Isolation of heart mitochondria. Rat heart mitochondria were isolated according to the method described for brain mitochondria by Voss *et al.*,[11] using a mannitol–sucrose medium. This medium contained 0·21 M mannitol, 0·075 M sucrose, 0·01 M Tris, and 0·1 mM EDTA. The final pH was 7·4.

Methods of assay. The polarographic determinations of oxidative phosphorylation were made with an oxygen electrode as described by Voss *et al.*[12] The P/O ratios were calculated as ADP/O ratios according to the method of Chance and Williams[13] for assaying oxidative phosphorylation. The ADP/O ratios were calculated from the uptake of oxygen in microatoms per liter during the active state of respiration and the molar concentration of ADP added. The respiratory control coefficients (RC) were calculated as the ratios of respiratory rates with ADP and the control respiration after ADP was consumed. All the spectrophotometric determinations were made in a Beckman DB spectrophotometer. Protein was determined by the method of Lowry *et al.*[14]

Particles with NADH-oxidase activity. Submitochondrial particles with NADH-oxidase activity[15] were prepared by adopting the procedure described by Kielley and Kielley[16] for preparation of ATPase. Mitochondria from 40 *g* of liver were suspended in 55 ml of 0·003 M phosphate buffer, pH 7·5, and treated for 2 min in the cold with a Sorvall Ommi-mixer homogenizer with micro-attachment (24,000 rev/min). Large particles were removed by centrifugation in a model L spinco ultracentrifuge, No. 40 Rotor at 20,000 *g* for 10 min. The supernatant solution was recentrifuged at 105,000 *g* for 30 min, and the sediment was resuspended in 0·003 M phosphate buffer, pH 7·5. The activity of this suspension was measured with an oxygen electrode. The assay medium contained: phosphate buffer, 0·05 M, pH 7·5; NADH, $3{\cdot}3 \times 10^{-4}$ M; NADH-oxidase (3·2 mg protein); propranolol, $4{\cdot}81 \times 10^{-4}$ M; in a total volume of 2·4 ml.

NADH-cytochrome c reductase. Mitochondria were isolated by the method described by Crane *et al.*[17] NADH-cytochrome *c* reductase (Complex I–III of the respiratory chain) was prepared by the method of Hatefi and Rieske.[18] The reduction of ferricytochrome *c* by NADH was measured spectrophotometrically at 550 mμ, using the following reaction mixture: to each of two 1-ml quartz cuvettes were added 0·02 ml of 1·0 M phosphate buffer, pH 8; 0·02 ml of 0·1 M sodium azide; 0·06 ml of 1% ferricytochrome *c*; 0·06 ml of 0·001 M EDTA, and water to a total volume of 1·0 ml. A few minutes prior to assay, the enzyme was diluted to a concentration of 0·1 to 1·0 ml/ml in an aqueous medium that is 0·05 M in tris–HCl, pH 8; 0·67 M in sucrose; and 0·001 M in histidine. The reaction was started by an introduction of 0·01 or 0·02 ml of the diluted enzyme into the sample cuvette. To test the action of the drug, $1{\cdot}1 \times 10^{-4}$ M propranolol was introduced in both cuvettes.

Heart mitochondrial transporting particles (inner membrane). Beef heart mitochondria were prepared by the method of Crane *et al.*[17] Isolation of EP_1 particles was made by the method of Kopaczyk[19] and the NADH-oxidase activity was measured polarographically. The assay medium contained: 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 (3·1 mg protein) and NADH, $3{\cdot}3 \times 10^{-4}$ M; in a total volume of 2·4 ml.

Materials. Biochemical reagents were obtained from the Sigma Chemical Co., St. Louis, Mo. Propranolol (Imperial Chemical Industries, Wilmslow Cheshire, Great

TABLE 1. EFFECT OF PROPRANOLOL ON OXIDATIVE PHOSPHORYLATION IN RAT HEART MITOCHONDRIA*

Experiments + final molarity in samples	Glutamate		α-Ketoglutarate		Malate + pyruvate		Succinate	
	RC	ADP/O	RC	ADP/O	RC	ADP/O	RC	ADP/O
Control	7·5	3·4	3·1	3·1	5·0	3·2	2·1	1·8
Propranolol:								
$4{\cdot}81 \times 10^{-4}$ M	5·0	2·9	2·6	2·9	3·2	2·9	2·2	1·8
$9{\cdot}62 \times 10^{-4}$ M	3·9	2·3	2·3	2·1	2·1	2·2	1·8	1·6
$1{\cdot}44 \times 10^{-3}$ M	2·5		1·0		1·0		1·8	1·4

* System contained: 2·2 ml of aerobic medium (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); 0·2 ml (4 mg of protein) of mitochondrial suspension; 10 μmoles substrates, and 235 μmoles of ADP, RC and ADP/O ratios are defined under Methods.

TABLE 2. ACTION OF PROPRANOLOL ON THE UNCOUPLING EFFECT OF 2,4-DINITROPHENOL*

Substrates	Respiration	2,4-DNP
Glutamate		
Control	0·21	0·71
Propranolol	0·15	0·23
α-Ketoglutarate		
Control	0·17	0·39
Propranolol	0·10	0·22
L-Malate + pyruvate		
Control	0·23	0·56
Propranolol	0·14	0·27

* System contained: 2·2 ml of aerobic medium (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); 0·2 ml (4 mg of protein) of mitochondrial suspension; 10 μmoles substrates; 2,4-dinitrophenol $2{\cdot}10^{-4}$ M; and propranolol, $1{\cdot}44 \times 10^{-3}$ M. Figures are expressed as oxygen uptake in micromoles of $O_2 \cdot sec^{-1} \cdot liter^{-1}$.

TABLE 3. EFFECT OF PROPRANOLOL ON ENZYMATIC ACTIVITIES*

Activity measured	Inhibitor concn (M)	Inhibition (%)
NADH-oxidase	$2{\cdot}88 \times 10^{-4}$	82
NADH-cytochrome *c* reductase	$1{\cdot}1 \times 10^{-5}$	36
	$5{\cdot}5 \times 10^{-5}$	90
	$1{\cdot}1 \times 10^{-4}$	98
Elementary particles-EP_1 (NADH-oxidase)	$1{\cdot}44 \times 10^{-4}$	61
	$2{\cdot}88 \times 10^{-4}$	86

* Assays described under Methods.

Britain) was generously supplied by Dr. Gastão P. Cunha from the Hospital of the University of Paraná. All other chemicals were of analytical reagent grade quality.

RESULTS

Effect of propranolol on the mitochondrial oxidative phosphorylation. The effect of propranolol on oxidative phosphorylation in rat heart mitochondria was assayed polarographically using oxygen electrode (Table 1).

Action of propranolol on the uncoupling effect of 2,4-*dinitrophenol.* Polarographic experiments were carried out in order to see the possible effect of propranolol on the property which 2,4-dinitrophenol displays in stimulating the respiration of mitochondria. Table 2 shows the results of these experiments.

Effect of propranolol on enzymatic activities. Since the oxidation of NAD^+-linked substrates was inhibited, it was assumed that propranolol would act probably at the NADH-oxidase segment of the chain (Table 3). It was felt that it was possible to locate further the site of blockade by preparing submitochondrial particles with NADH-oxidase activity, NADH-cytochrome *c* reductase and NADH-oxidase from heart mitochondrial electron transporting particles (inner membrane).

DISCUSSION

The stimulation by catecholamines of the enzyme adenyl cyclase which catalyzes the formation of 3′,5′-AMP, as well as the inhibition of this effect by adrenergic blocking agents, has been demonstrated in broken cell preparations from dog heart.[2] Furthermore, an increase in concentration of 3′,5′-AMP has been demonstrated in intact heart *in vivo*[20] and *in vitro*[21] subsequent to the addition of epinephrine. This effect is also blocked by beta-adrenergic antagonists and possibly by parasympathetic stimulation.[22]

Several points related to the cellular location of the adenyl cyclase system may be worth mentioning. Studies of the subcellular distribution of adenyl cyclase do not appear to exclude the possibility that a fraction of the enzyme is associated with intracellular organelles. For example, cyclase activity of brain was found in all particulate fractions.[23] According to Sutherland *et al.*,[21] adenyl cyclase was associated with particulate material in broken cells preparations, the origin of which was not mitochondria or microsomes; the cellular source of the particulate material is unknown, but it may be derived from cell membranes or from nuclei.

In the present report, using isolated rat heart mitochondria, it has been found that increasing amounts of propranolol inhibit the respiratory control coefficient when glutamate, α-ketoglutarate and L-malate coupled with pyruvate (Table 1), which is an indication that the oxidative phosphorylation is increasingly inhibited after the uncoupling of the mitochondria. Sobel *et al.*[9] using propranolol (10^{-3} M) have shown that the oxidation of malate-pyruvate by normal heart mitochondria was not affected by this adrenergic blocking agent. Under the conditions of the present experiments and using lower concentrations of propranolol ($4{\cdot}81 \times 10^{-4}$ M and $9{\cdot}62 \times 10^{-4}$ M), we have been able to obtain about 50 per cent inhibition when NAD^+-linked substrates were used. Concerning the effect of propranolol ($1{\cdot}44 \times 10^{-3}$ M) on the respiratory control coefficient when succinate was the substrate, Table 1 shows that

only an inhibition of 14 per cent was obtained. Similar effect could be observed on the oxidative phosphorylation.

The fact that 2,4-DNP did not release respiration after propranolol is indicative of the latter's possible action more proximal to the electron transport chain than 2,4-DNP. Propranolol, even in high concentrations ($1{\cdot}44 \times 10^{-3}$ M), cannot completely inhibit NADH-linked electron transport as does rotenone (Table 2).

The evidence that propranolol has a selective action on NADH-oxidase activity is shown in Table 3. With three enzymatic preparations we have been able to obtain almost complete inhibition of NADH oxidation. With the first preparation, the oxidation of NADH was reversed with the addition of methylene blue ($4{\cdot}1 \times 10^{-5}$ M). Since the oxidation of NAD^+-linked substrates and NADH oxidation were inhibited, it was assumed that this beta-bocking agent acts in the NADH-oxidase segment of the chain, its site of action being localized between NAD^+ and the flavoprotein.

In order to eliminate the possibility that the inhibition by propranolol may be due to a possible blockade of cyclic AMP formation, the inhibition by propranolol was studied to determine if it could be overcome by dibutyryl cyclic AMP (DBc AMP),[24] the fat-soluble derivative of cyclic AMP. DBc AMP used in concentrations of 10^{-4}, 2×10^{-4} and 4×10^{-4} M, in system similar to that one of Table 1, does not overcome the inhibition caused by propranolol with any one substrate used. Murad and Vaughan[25] showed that propranolol (2×10^{-5} M) had no appreciable effect on cyclic 3′,5′-AMP formation in washed particulate preparations of adenyl cyclase from rat heart. The effect of propranolol on mitochondrial preparations seems to be independent of a blockade of catecholamine activity at the cyclase level.

It is possible that the low concentrations of propranolol used in the experiments may still be higher than those found *in vivo*. Therefore, no extrapolations from the present data to the *in vivo* state can be made.

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