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# COORDINATE INCREASES AND DECREASES IN MITOCHONDRIAL RNA AND ATP SYNTHESES PRODUCED BY PROPRANOLOL AND RIFAMPICIN

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**Abstract**—A variety of compounds were examined for their capacity to alter RNA synthesis in isolated rat cardiac and hepatic mitochondria. The beta-adrenergic blocking agents propranolol and butoxamine, and the antiarrhythmic agent quinidine, produced a concentration-dependent stimulation of RNA synthesis in cardiac and hepatic mitochondria. In contrast, the antitubercular antibiotic rifampicin produced a concentration-dependent inhibition of RNA synthesis in cardiac and hepatic mitochondria. Propranolol, as a representative compound which stimulated RNA synthesis, was also found to stimulate ATP synthesis in isolated mitochondria, whereas rifampicin inhibited ATP synthesis. Coordinate increases and decreases in RNA and ATP syntheses suggest that agents which stimulate or inhibit RNA synthesis may rapidly alter ATP synthesis. This finding is consistent with the rapid turn-over of mitochondrial RNA with a messenger function (1.4 and 3.3 min in isolated rat cardiac and hepatic mitochondria), and it suggests that mitochondrial RNA must continue to be synthesized to maintain inner membrane systems required for ATP synthesis. Stimulation of RNA and ATP syntheses by propranolol through membrane stabilization or other actions represents a heretofore unrecognized action of propranolol which may contribute to its beneficial therapeutic effects.

Propranolol is a nonselective beta-adrenergic blocking agent used in the clinical management of arrhythmias, hypertension, and angina pectoris [1]. Propranolol has been demonstrated to be effective in reducing the recurrence of myocardial infarction [1]. Propranolol may alter cellular metabolism, an effect produced by the blockade of beta-adrenergic receptor-mediated activation of adenyl cyclase activity [2]. Propranolol has been reported to have a membrane-stabilizing effect, alternately described as a local anesthetic or quinidine-like action [1]. In contrast to propranolol, butoxamine is a somewhat selective $beta_2$-adrenergic antagonist, blocking vasodilation produced by $beta_2$-adrenergic agonist activity while producing little antagonism of $beta_1$-adrenergic agonist action on the heart.

Rifampicin is an antitubercular antibiotic that acts as a selective inhibitor of bacterial transcription after binding to bacterial RNA polymerase [3]. While it does not inhibit eukaryotic transcription, it has been shown to produce direct inhibitory effects on eukaryotic protein synthesis [4]. Rifampicin has also been shown to act as a direct inhibitor of RNA synthesis in mitochondria isolated from eukaryotic cells [5].

In this paper we report coordinate increases in RNA and ATP syntheses in mitochondria isolated from rat heart and liver in the presence of propranolol, butoxamine and quinidine. Conversely, we show coordinate decreases in RNA and ATP syntheses produced by rifampicin. These findings suggest that agents which alter mitochondrial RNA synthesis may produce alterations in ATP synthesis, and that high concentrations of propranolol and quinidine may have previously unrecognized actions in stimulating mitochondrial RNA and ATP production through stabilization of mitochondrial membranes or through other actions.

## MATERIALS AND METHODS

*Materials.* Propranolol, butoxamine, quinidine, ouabain, digoxin, procaine, aminophylline, caffeine, cyclic nucleotides, AMP, UMP, sucrose, D-mannitol, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), crystalline bovine serum albumin (BSA), trichloroacetic acid (TCA), $Na_2P_4O_7$ and other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO. Ammonium molybdate was purchased from Mallinckrodt Chemical Works, St. Louis, MO. Scintanalyzed toluene, benzene, and isobutanol were obtained from the Fisher Scientific Co., Houston, TX, and Soluene, Insta-Gel, 2,5-diphenyloxazole (PPO), 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) from the Packard Instrument Co., Downers Grove, IL. ATP, ADP, CTP, GTP and UTP came from P-L Biochemicals, Milwaukee, WI. [5-$^3$H]Uridine-5′-triphosphate (20 Ci/mmol) as the sodium salt, and [$^{32}$P]phosphoric acid (200 Ci/mmol) were supplied by Amersham/Searle, Arlington Heights, IL.

*Isolation of mitochondria.* Sprague–Dawley rats, obtained from Simonsen Laboratories, Gilroy, CA, were used as breeding stock. Rat cardiac and hepatic mitochondria were isolated using the technique of Schnaitman and Greenawalt [6]. BSA was used in the buffers to prevent the uncoupling of mitochondria by

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fatty acids released during preparation. Four male rats (60–150 g) were fasted for 12 hr to reduce hepatic glycogen, which interfered with the isolation of hepatic mitochondria. Animals were stunned and decapitated, and the hearts or livers were pooled. Organs were washed, minced and homogenized in cold sucrose–mannitol–HEPES buffer (SMH: 220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES and 0.5 mg/ml crystalline BSA, pH 7.4 at 0°). Homogenates were centrifuged at 560 *g* for 15 min at 4° and supernatant fractions at 700 *g* for 15 min. The crude mitochondrial pellet was gently suspended in one-half the original volume of SMH and centrifuged at 700 *g* for 15 min. The pellet was then suspended in one-fourth the original volume of SMH and centrifuged at 700 *g* for 15 min. The purified mitochondrial pellet was suspended in SMH without BSA in the proportion of 1.0 ml SMH/organ. The concentration of hepatic mitochondrial protein obtained using the Bio-Rad protein assay was consistently close to 40 mg/ml (mean ± SEM = 43.82 ± 1.69 mg mitochondrial protein/ml; 20 determinations). Mitochondrial protein obtained from cardiac tissue was lower than that obtained from hepatic tissue.

*Assays for mitochondrial RNA synthesis.* Assays for mitochondrial RNA synthesis [5] were performed using approximately 1.0 mg mitochondrial protein, ribonucleoside triphosphates (ATP, CTP and GTP: 1.6 mM; UTP: 0.16 mM), 2.5 μCi [$^3$H]UTP (1 Ci/mmol) in a volume of 0.25 ml SMH, pH 7.4, at 30°. UTP was not limiting in the assay. Incorporations were allowed to proceed for 15 min at 30° and were quenched by the addition of 0.25 ml SMH containing 100 mM $Na_2P_4O_7$ to reduce nonspecific label binding. Immediately afterward, 1.0 ml of 10% TCA containing $Na_2P_4O_7$ was added to precipitate macromolecular material. The samples were vortexed, and 5% TCA was added to a final volume of 2.5 ml. Tubes were held on ice for 10 min and then centrifuged at 17,300 *g* for 10 min at 4°. The supernatant fraction was aspirated off, and the pellet was washed twice by dissolving it in 1.0 ml of 0.1 M NaOH followed by reprecipitation with TCA. In controls for nonspecific label binding, TCA was added immediately after mitochondria to cold buffer containing label. Tubes containing washed pellets were inverted and dried overnight at 2°. Pellets were dissolved in 0.5 ml Soluene and were taken up in a toluene scintillation mixture for counting at 30% efficiency. Control values representing background label binding were subtracted from experimental values. The automatic external standard of the scintillation spectrometer was used to correct for chemical quenching and for quenching by rifampicin that remained bound to the final pellet and imparted an orange color to the counting solution.

*Assay conditions for ATP synthesis.* To measure ATP synthesis, 1.0 mg mitochondrial protein was pipetted into 1.0 ml SMH containing 1.0 mM ADP and 0.5 mM $KH_2PO_4$–$K_2HPO_4$ labeled with $^{32}$P (sp. act. 200,000 cpm/ml assay medium). Tubes were incubated for 15 min at 30°, and assays were quenched by the addition of 1.0 ml of cold 20% TCA. Unincorporated inorganic phosphate containing label was removed from esterified $^{32}$P as follows [7]. Acetone (1.0 ml) was added to 1.0 ml of the assay and, after mixing, the sample was permitted to stand for 10 min. Then 1.0 ml of isobenzene-saturated water was added, followed by 1.0 ml of water-saturated isobutanol–benzene. The mixture was vortexed to extract the acetone from the lower aqueous phase to the upper organic phase. Next, 0.25 ml of 5% ammonium molybdate in 4 N $H_2SO_4$ was slowly added down the inside wall of the tube so that a layer of yellowish-white molybdate appeared in the lower part of the aqueous phase. The tube was then gently swirled to mix the molybdate with the aqueous phase. The tubes were permitted to stand for 10 min, and the neutral phosphomolybdate particles were extracted to the organic phase by vortexing. The organic phase was carefully suctioned off. One drop of 40 mM $KH_2PO_4$ was added as a carrier to the aqueous phase and, after shaking, 7.0 ml of water-saturated isobutanol–benzene was added. After vortexing and standing for 5 min, the organic phase was again removed. The steps in which $KH_2PO_4$ and water-saturated isobutanol–benzene were added were repeated twice to ensure complete extraction of the free inorganic phosphate remaining. One-half milliliter of the aqueous phase was then added to 5.0 ml Insta-gel for counting at an efficiency of 71%.

## RESULTS

Isolated rat cardiac and hepatic mitochondria were used to examine compounds for their capacity to alter mitochondrial [$^3$H]UTP incorporation used as a measure of mitochondrial RNA synthesis. The incorporation of [$^3$H]UTP was found to be essentially linear in cardiac mitochondria for at least 60 min and in hepatic mitochondria for at least 45 min. The capacity of specific agents to alter mitochondrial RNA synthesis was then examined during a 15-min assay period, well within the linear [$^3$H]UTP incorporation period. In Table 1 we have listed those agents that had no, or minimal, effects on mitochondrial RNA synthesis, that inhibited RNA synthesis, and that stimulated RNA synthesis.

We next examined the capacity of representative agents which stimulated (propranolol) and inhibited (rifampicin) mitochondrial RNA synthesis to alter mitochondrial ATP synthesis (Table 2). Propranolol produced a dose-dependent stimulation of ATP synthesis in cardiac mitochondria and stimulated ATP synthesis in hepatic mitochondria. Conversely, rifampicin produced a dose-dependent decrease in ATP synthesis in cardiac mitochondria and inhibited ATP synthesis in hepatic mitochondria. These data suggest that agents which alter mitochondrial RNA synthesis may also alter ATP synthesis in a coordinate fashion.

## DISCUSSION

In our survey of the effects of chemical agents on mitochondrial [$^3$H]UTP incorporation, ouabain, aminophylline, caffeine and the catecholamines epinephrine, norepinephrine and L-isoproterenol were without effect (data not shown). The cyclic nucleotides, cAMP, cCMP, cGMP and cUMP, and the nucleotides GMP and CMP, had no effect on mitochondrial RNA synthesis, whereas AMP, ADP

Table 1. Effects of various agents on [$^3$H]UTP incorporation into cardiac and hepatic mitochondria*

| Drug (100 μg/ml) | Mitochondrial [$^3$H]UMP incorporation (dpm) | | | | | |
|---|---|---|---|---|---|---|
| | Cardiac | | | Hepatic | | |
| | −Drug | +Drug | $\frac{+\text{Drug}}{-\text{Drug}} \times 100$ (%) | −Drug | +Drug | $\frac{+\text{Drug}}{-\text{Drug}} \times 100$ (%) |
| I. Agents with no effect or a questionable effect on [$^3$H]UMP incorporation: | | | | | | |
| Digoxin† | 5,190 ± 150 | 4,990 ± 120 | 96 | 1,390 ± 10 | 1,400 ± 50 | 101 |
| Cyclic AMP | 5,730 ± 130 | 5,520 ± 120 | 96 | 1,870 ± 20 | 1,860 ± 60 | 100 |
| Cyclic UMP | 15,450 ± 70 | 16,000 ± 120 | 104 | 4,460 ± 50 | 4,480 ± 50 | 100 |
| Cyclic GMP | 8,000 ± 50 | 8,230 ± 160 | 103 | 4,460 ± 50 | 4,390 ± 50 | 98 |
| Cyclic CMP | 15,450 ± 70 | 14,340 ± 180 | 93 | 4,460 ± 50 | 4,050 ± 80 | 91 |
| GMP | 12,270 ± 200 | 11,750 ± 130 | 96 | 2,290 ± 50 | 2,310 ± 30 | 101 |
| CMP | 8,000 ± 50 | 8,230 ± 100 | 103 | 2,290 ± 50 | 2,240 ± 70 | 98 |
| Procaine† | 5,730 ± 130 | 5,570 ± 100 | 97 | 1,870 ± 20 | 2,070 ± 40 | 111 |
| II. Agents that inhibit [$^3$H]UMP incorporation: | | | | | | |
| AMP | 8,000 ± 50 | 5,510 ± 140 | 69 | 2,970 ± 60 | 2,470 ± 70 | 83 |
| ADP | 15,560 ± 320 | 7,230 ± 370 | 47 | 3,210 ± 90 | 2,350 ± 50 | 73 |
| UMP | 12,270 ± 200 | 9,720 ± 590 | 79 | 2,290 ± 50 | 1,820 ± 150 | 79 |
| Rifampicin† | | Not determined | | 1,750 ± 40 | 400 ± 20 | 23 |
| III. Agents that stimulate [$^3$H]UMP incorporation: | | | | | | |
| Propranolol | 10,670 ± 90 | 14,390 ± 1,380 | 135 | 2,970 ± 60 | 8,680 ± 60 | 292 |
| Butoxamine | 12,940 ± 250 | 17,120 ± 150 | 132 | 2,620 ± 50 | 3,590 ± 80 | 137 |
| Quinidine | 15,560 ± 320 | 19,430 ± 350 | 125 | 3,210 ± 90 | 4,910 ± 220 | 153 |

* Cardiac and hepatic mitochondria were isolated from 60–150 g male Sprague–Dawley rats. Incubations were performed in SMH buffer (pH 7.4) for 15 min at 30° with 0.8 to 1.3 mg mitochondrial protein in the presence of 2.5 μCi [$^3$H]UTP (20 Ci/mmol). Values are the averages of four replicates ± SE. Different control values (without drugs) represent values obtained using different mitochondrial preparations. Average counting efficiency: 30%. Drugs were added from concentrated stock solutions made up in distilled water except for the exceptions noted.

† Drugs were dissolved in a small volume of absolute ethanol. Ethanol was added to control assays in amounts equal to the final concentrations in the experimental assays.

and UMP depressed RNA synthesis (Table 1). Decreases in the incorporation of [$^3$H]UTP induced by ADP and AMP were dose dependent (data not shown). This finding is consistent with the capacity of adenine nucleotides to exchange with ATP via adenine nucleotide translocase in the mitochondrial membrane [8]. Extramitochondrial ADP is exchanged for intramitochondrial ATP, reducing available triphosphate levels for [$^3$H]UTP incorporation. UMP would also provide a substrate for phosphorylation and reduce the specific activity of intramitochondrial [$^3$H]UTP, reducing apparent label incorporation.

Of primary interest in Table 1 are the findings that the beta-blocking agents propranolol and butoxamine stimulated mitochondrial RNA synthesis. The antiarrhythmic drug quinidine also stimulated RNA synthesis, but it was less potent than propranolol. Those agents which stimulated mitochondrial RNA synthesis did so in a dose-dependent manner, and they stimulated hepatic RNA synthesis more strongly than cardiac RNA synthesis.

Table 2 demonstrates that there was a coordinate increase in ATP synthesis in cardiac and hepatic mitochondria in the presence of propranolol. Clinically effective plasma levels of propranolol are 15–90 ng/ml for antianginal effects, 120 ng/ml for a 50% reduction in exercise-induced cardioacceleration, and up to 1 μg/ml to control resistant ventricular arrhythmias [1]. The dose-dependent effects of propranolol on RNA and ATP syntheses shown in Tables 1 and 2 occurred at concentrations of propranolol ranging from 10 to 100 μg/ml (approximately 0.03 to 0.33 mM propranolol). These concentrations of propranolol are associated with the direct myocardial depression characteristic of drugs producing membrane stabilization, e.g. direct myocardial depressant effects have been observed in dogs when 1–2 mg/kg propranolol is given intravenously [9–12]. Concentrations of propranolol required to demonstrate membrane-stabilizing effects are, therefore, at least 100-fold higher than the blood levels associated with the inhibition of exercise tachycardia [13–15]. However, membrane stabilization may be of some importance when very high daily doses of propranolol are administered clinically (e.g. up to 640 mg [16]).

The mechanism of the direct depressant action of

Table 2. Effects of rifampicin and propranolol on ATP synthesis* in isolated rat cardiac and hepatic mitochondria†

| Drug concentration (μg/ml) | I. Cardiac mitochondria | | | |
|---|---|---|---|---|
| | Incorporation (cpm/mg protein) | | | |
| | Propranolol | % of Control | Rifampicin | % of Control |
| Expt. 1 0 | 65,280 | 100 | 65,280 | 100 |
| 10 | 68,000 | 104 | 54,270 | 83 |
| 25 | 82,000 | 126 | 47,060 | 72 |
| 50 | 92,160 | 141 | 51,190 | 78 |
| 100 | 94,080 | 144 | 47,720 | 73 |
| Expt. 2 0 | 145,130 | 100 | 145,130 | 100 |
| 100 | 192,030 | 132 | 124,700 | 86 |
| | II. Hepatic mitochondria | | | |
| Expt. 1 0 | 72,910 | 100 | 72,910 | 100 |
| 100 | 95,090 | 130 | 53,500 | 73 |

* ATP formation was measured by incubating 1.0 mg of mitochondrial protein at 30° for 15 min in 1.0 ml medium containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES buffer, 1 mM ADP and 0.5 mM $KH_2PO_4$-$K_2HPO_4$ labeled with 200,000 cpm/ml $^{32}P$. Samples were prepared for counting as described in Materials and Methods. Values shown were corrected for nonspecific $^{32}P$ binding (approximately 1100 cpm). Counting efficiency was 71%.

† Mitochondria were prepared from 110–150 g male Sprague–Dawley rats as described in Materials and Methods.

propranolol through membrane stabilization or local anesthetic effect is thought to involve an inhibition of the binding and uptake of $Ca^{2+}$ and an inhibition of $Ca^{2+}$ and $Mg^{2+}$-ATPase activities. Propranolol (1–5 mM) depresses rat cardiac sarcolemmal $Na^+$-$K^+$-ATPase, $Mg^{2+}$-ATPase and $Ca^{2+}$-ATPase [17]. Propranolol at 5 mM inhibits mitochondrial ATPase activities by approximately one-third [17]. Propranolol has been shown to have a $K_I$ of approximately 0.3 mM for competitive inhibition of $Ca^{2+}$ binding and uptake by dog cardiac microsomes [18]. Perfusion of rat hearts with 0.2 to 1.0 mM propranolol decreases contractile force and calcium accumulation by microsomes and mitochondria; propranolol at 0.5 and 1 mM depresses rat heart microsomal calcium uptake by 40 and 63%, respectively, and mitochondrial calcium uptake by 10 and 28% [19]. Quinidine has also been reported to inhibit cardiac microsomal calcium binding and uptake [20]. As $Ca^{2+}$- and $Mg^{2+}$-ATPase are believed to regulate the movement of $Ca^{2+}$ across membranes, propranolol probably reduces ion uptake by reducing membrane ATPase activity.

Mitochondria are known to accumulate calcium against a concentration gradient via an electrophoretic uniporter [21, 22]. Transport is dependent upon an energy source, which may be supplied by exogenously added ATP or a metabolic substrate [21, 23]. Mitochondrial uptake of calcium to 100–200 nmol/mg mitochondrial protein occurs at the expense of oxidative phosphorylation [24, 25]. The buffers used in our assays contained no added calcium, but calcium may have been released from damaged mitochondria in sufficient amounts to compete with oxidative phosphorylation. While the membrane stabilization by propranolol may inhibit membrane ATPases, it did not inhibit ATP synthetase as increasing propranolol concentrations up to 100 μg/ml increased ATP synthesis (Table 2).

In contrast to propranolol, the antibiotic rifampicin coordinately inhibited both RNA and ATP syntheses in isolated cardiac and hepatic mitochondria (Tables 1 and 2). Rat liver mitochondrial RNA products synthesized *in vitro* are similar in number and size to those synthesized *in vivo* [26–28]. Gamble and McClure [29] have shown that bovine heart mitochondrial mRNA has a half-life of 1.4 min. Mitochondrial transcription products include lipophilic subunits of cytochromes *b*, *c* and $c_1$, cytochrome *c* oxidase and ATPase [26–28, 30–32]. Ethidium bromide, an agent that disrupts transcription by intercalation, produces an inhibition of oxidative phosphorylation in mitochondria [33], while yeast petite mutants have defective respiratory and phosphorylating capacities [28].

We have reported previously that rifampicin inhibits the synthesis of RNA with a messenger function in isolated rat hepatic mitochondria, permitting an estimate of the turn-over of mitochondrial mRNA of 3.3 min [5]. In view of the rapid turn-over of mitochondrial RNA, we propose that blockade of mitochondrial RNA synthesis rapidly produces reductions in ATP synthesis due to a failure to continue protein synthesis involved in the maintenance of inner membrane enzyme systems, ion fluxes and oxidative phosphorylation. Conversely, if propranolol increases mitochondrial RNA synthesis, ATP synthesis could be increased by increasing the concentration of respiratory enzyme complexes in the inner membranes of mitochondria. For this to occur,

concentrations of the subunits of the inner membrane enzymes produced in mitochondria would have to be limiting, and mitochondria would have to contain pools of enzyme subunits that are produced on cytoplasmic ribosomes. Cytoplasmically produced proteins have been shown to stimulate mitochondrial DNA and protein syntheses [32, 34]; therefore, regulatory molecules may be present in isolated mitochondria and produce increases in transcription under appropriate conditions. However, it may also be argued that propranolol stabilizes the mitochondrial membrane and results in increased ATP synthesis, which in turn increases RNA synthesis due to an increased energy charge. In either case, elevated levels of agents which act to stabilize mitochondrial membranes result in increased ATP synthesis, a heretofore unrecognized property of these agents and one that could be related to their therapeutic effects.

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