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INFLUENCE OF THYROID AND GROWTH HORMONE STATUS ON THE RATE OF REGULATED Ca^{2+} EFFLUX FROM RAT LIVER MITOCHONDRIA

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SUMMARY: Measurements were made of Ca^{2+} efflux from liver mitochondria of normal, surgically thyroparathyroidectomized and hypophysectomized rats. Release of Ca^{2+} from preloaded mitochondria was initiated by the addition of ruthenium red, which blocks Ca^{2+} influx, and oxaloacetate, which stimulates Ca^{2+} efflux by bringing the mitochondrial pyridine nucleotides to a relatively oxidized steady-state. The time required to release a small amount of Ca^{2+} from mitochondria isolated from thyroparathyroidectomized or hypophysectomized rats was far longer than that observed with mitochondria from normal animals. Treatment of such rats with either triiodothyronine or bovine growth hormone for 3-4 days resulted in restoration of Ca^{2+} efflux to a near normal level.

INTRODUCTION

Intracellular Ca^{2+} plays an important role in the regulation of many cellular processes and often acts as a second messenger for extracellular stimuli (1). The distribution of intracellular Ca^{2+} in some tissues, such as liver, is regulated in part by energy-linked mitochondrial Ca^{2+} transport (2). Both the uptake and release of Ca^{2+} by isolated rat liver mitochondria can be affected by circulating levels of a number of hormones, including epine-

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ABBREVIATIONS USED: α-GPDH, alpha-glycerophosphate dehydrogenase; bGH, bovine growth hormone; con, control; EGTA, ethylene glycol bis(β-amino ethyl ester)N,N,N^1,N^1-tetraacetic acid; GH, growth hormone; HEPES, 4-(2-hydroethyl)-1-piperazinethanesulfonic acid; hypox, hypophysectomized; OAA, oxaloacetate; RR, ruthenium red; T3, triiodothyronine; TMPD, N,N,N^1,N^1-tetramethylphenylenediamine; Tx, thyroparathyroidectomized

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phrine and glucagon (3,4) and triiodothyronine (T3) (5). Mitochondrial Ca^{2+} transport is also influenced by many intracellular factors, including energy state, as indicated by the sensitivity of mitochondrial Ca^{2+} efflux to adenine nucleotides (6) and the oxidation-reduction state of the intramitochondrial pyridine nucleotides (7,8). Evidence is presented here that thyroid and growth hormones profoundly affect rat liver mitochondrial Ca^{2+} efflux evoked by the oxidized state of mitochondrial NAD(P)H. This provides an example of how extra- and intracellular modes of regulating Ca^{2+} transport can be integrated. A preliminary report of some of these findings has been presented (9).

MATERIAL AND METHODS

Animals and isolation of liver mitochondria. Male thyroparathyroidectomized rats (Tx) and normal control rats (Con) were obtained from Charles River (CD strain) and weighed 50-70 gm on arrival. After being maintained on a low iodine diet for 2 weeks, each rat was given 100 µCi ^{131}I intraperitoneally (i.p.) and thereafter kept on a normal laboratory chow diet for at least 6 weeks. Both Tx and Con animals received 0.85% $CaCl_2$ in their drinking water. The Tx rats weighed 140-195 gm when used and the Con rats 250-300 gm. Hypophysectomized (hypox) male rats obtained from the same supplier weighed 75-100 gm. They received no $CaCl_2$ in their drinking water and were used within 2 months of surgery. Target organs for pituitary hormones were markedly reduced in weight in these animals. T3 or bovine growth hormone (bGH) were dissolved in 0.01 N NaOH and administered i.p. Paired experimental and solvent injected control animals were fasted overnight before removal of livers and isolation of mitochondria. Mitochondria from the livers of the paired animals were prepared at the same time by the method of Schneider (10) in unbuffered 0.25 M sucrose. Mitochondrial protein was determined according to Lowry et al. (11) using bovine serum albumin as the standard.

Assays. Mitochondrial oxygen consumption was measured in a closed glass reaction vessel at 25°C with a Clark oxygen electrode. Mitochondria were suspended at 1 mg protein/ml in medium containing 125 mM sucrose, 60 mM KCl, 3 mM K-HEPES (pH 7.1), 1 mM K-phosphate, 0.5 mM K-EGTA, 5 mM K-succinate and 4 µM rotenone. Respiratory control ratios were determined as described by Estabrook (12). Mitochondrial α-glycerophosphate dehydrogenase (α-GPDH) activity was determined by the method of Lee and Lardy (13) and served as a marker for the effect of T3. Total mitochondrial Ca present in 0.1 N HCl extracts of isolated mitochondria was determined by atomic absorption. Changes in the Ca^{2+} activity of mitochondrial suspensions were measured at 25°C with a Ca^{2+}-selective electrode (Radiometer F2112 Calcium Selectrode) using a Thomas combination glass electrode (Model 4094-L25) as reference. The output from the electrode was amplified through a Sargent-Welch High Impedance Accessory and fed into a Bausch and Lomb Model VOM 7 strip-chart recorder.

Materials. T3 was obtained from Calbiochem and bGH was the gift of Dr. M. Sonenberg, Sloan-Kettering Institute. Ruthenium red (RR) was purified according to the method of Luft (14).

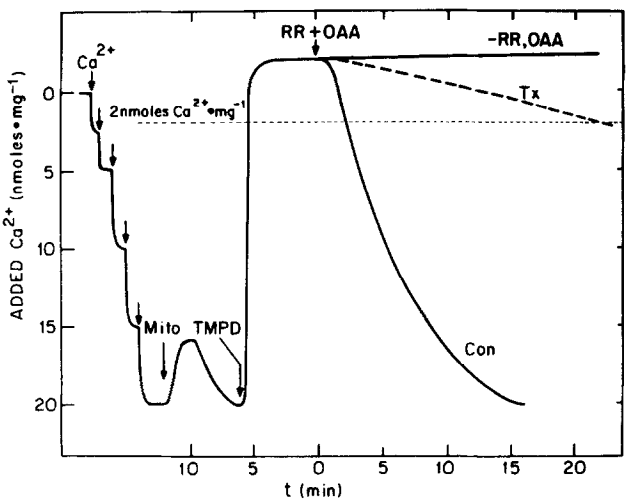

Fig. 1 Respiration-dependent mitochondrial Ca^{2+} uptake and oxaloacetate-stimulated ruthenium red-insensitive Ca^{2+} efflux.
Rat liver mitochondria (mito) from either euthyroid control rats (con) or Tx rats were added at a concentration of 2 mg protein/ml to 3 ml of medium containing 125 mM sucrose, 60 mM KCL, 3 mM K-HEPES, (pH 7.1), 0.2 mM K-phosphate, 4.0 mM ascorbic acid, 4 μM rotenone, and 0.2 μM antimycin A. 20 nmoles of $Ca^{2+}\cdot mg^{-1}$ mitochondrial protein had previously been added in small aliquots to calibrate the response of the electrode. The suspension was maintained at 25°C, stirred magnetically, and continuously exposed to a gentle stream of humidified O_2 to prevent anaerobiosis. Ca^{2+} uptake was initiated by the addition of the electron transport mediator TMPD (70 μM); efflux was initiated by the addition of 5 μM ruthenium red (RR) and 0.5 mM oxaloacetate (OAA). Measurements were made of the time taken after these additions for 10% of the added Ca^{2+} (2 nmoles$\cdot mg^{-1}$ protein) to be released. The logarithmic response of the Ca^{2+} electrode was linearized graphically.

RESULTS

The linearized Ca^{2+} electrode tracings shown in Fig. 1 describe a typical experiment in which respiration-dependent mitochondrial Ca^{2+} influx and oxaloacetate-stimulated RR-insensitive Ca^{2+} efflux were measured. Transient uptake of a small amount of Ca^{2+} was observed upon addition of mitochondria to a medium containing 20 nmoles $Ca^{2+}\cdot mg^{-1}$ protein and the "site 1 and 2" respiratory inhibitors rotenone and antimycin. Subsequent addition of the artificial "site 3" electron transport mediator TMPD in the presence of the electron donor ascorbate resulted in rapid uptake of the added Ca^{2+} as well as most of the Ca^{2+} present as a contaminant of the medium ($\sim$ 2 nmoles$\cdot mg^{-1}$ protein). Under these conditions no differences in the initial rate or extent of Ca^{2+} uptake by mitochondria from rats of different thyroid or growth hormone (GH) status were observed (results not shown).

Upon completion of mitochondrial Ca^{2+} uptake, the efflux of Ca^{2+} was initiated by the addition of RR, which blocks Ca^{2+} influx, and oxaloacetate (OAA), which stimulates the release of Ca^{2+} through the oxidization of mitochondrial pyridine nucleotides via the malate dehydrogenase reaction (7). After a lag period of approximately 0.5 min, relatively rapid net efflux of Ca^{2+} from liver mitochondria of Con rats occurred; 10% of the added Ca^{2+} (=2 nmoles•mg-1) + the contaminating Ca^{2+} being released within approximately 2.5 min after the addition of RR and OAA (Fig. 1). However, when the same additions were made to a suspension of mitochondria isolated from a paired Tx rat, it took almost 10-times longer for 10% of the Ca^{2+} load to be released. In other words, Ca^{2+} efflux from normal rat liver mitochondria was nearly 10-times faster than that from mitochondria of the Tx rat.

Significant differences in Ca^{2+} efflux were also apparent between mitochondria isolated from Con and hypox animals and between mitochondria from either Tx or hypox rats and similar animals that were injected with T3 or bGH for several days. A summary of these data, along with other pertinent information concerning the characteristics of each group of paired animals, is given in Table 1. In addition, mitochondria isolated from each rat were tested with the oxygen electrode for differences in generalized membrane permeability and energy coupling, as reflected by the respiratory control ratio (RCR) for oxidative phosphorylation (12). The pooled mean RCRs for mitochondria from Con animals was 3.7 ± 0.7 (s.d., n=10), for Tx animals it was 4.4 ± 0.8 (n=17), and for hypox animals it was 4.3 ± 0.7 (n=17). In addition, the mean RCRs for mitochondria from each of the T3 and bGH treated groups of animals was greater than 3.0 and did not correlate with differences in Ca^{2+} release times.

The mean values for the Ca^{2+} release time obtained from experiments such as those described by Fig. 1 are given in Row A of Table 1. These indicate that the average time required for release of 10% of the Ca^{2+} load from liver mitochondria of Tx rats was more than 15-times longer than that for normal rat liver mitochondria. The Tx rats also had abnormally low body weights and low mitochondrial α-GPDH activity, as expected of hypothyroid animals (Table

TABLE 1

Mitochondrial Ca^{2+} release times and accompanying data for rats of different thyroid and growth hormone status.

	Paired Animals	Ca^{2+} Release Time (min)	Body Weight (gm)	αGPDH Activity (ΔOD_{500nm} (x10) · min^{-1} · mg^{-1})	Mitochondrial Ca (nmoles · mg^{-1})
A	(6) Tx	42.4 ± 11.2[a]	125 ± 5	0.26 ± 0.08	12.6 ± 0.9
	Con	1.8 ± 0.4	278 ± 29	1.19 ± 0.26	23.3 ± 1.2
B	(6) Tx	16.7 ± 9.9[a]	165 ± 6	0.76 ± 0.16	20.8 ± 2.2
	Tx + T3	5.4 ± 3.9	141 ± 5	4.94 ± 0.44	20.2 ± 2.1
C	(6) Tx	23.6 ± 5.7[b]	138 ± 15	0.13 ± 0.04	11.6 ± 0.7
	Tx + bGH	10.2 ± 3.9	138 ± 9	0.13 ± 0.04	14.8 ± 1.3
D	(5) Hypox	20.2 ± 5.7[b]	70 ± 1	0.13 ± 0.02	22.7 ± 4.1
	Con	1.3 ± 0.1	224 ± 13	1.16 ± 0.16	22.6 ± 1.6
E	(6) Hypox	29.2 ± 8.0[a]	84 ± 6	0.70 ± 0.20	18.8 ± 2.3
	Hypox + T3	1.6 ± 0.4	35 ± 7	4.58 ± 0.41	18.7 ± 3.2
F	(5) Hypox	11.4 ± 3.3[b]	79 ± 5	0.12 ± 0.01	21.0 ± 3.1
	Hypox + T3 (low)	2.1 ± 0.2	78 ± 3	5.27 ± 0.64	30.0 ± 5.1
G	(5) Hypox + bGH	11.5 ± 5.0[b]	90 ± 11	0.45 ± 0.13	15.1 ± 0.5
	Hypox + bGH + T3	1.2 ± 0.1	89 ± 10	6.47 ± 0.74	18.1 ± 0.4

The numbers in parentheses are the numbers of animal pairs used for each study. Treatment of the rats was as described in the text and in the Materials and Methods section. All values listed are the means ± the standard error. The Ca^{2+} release time is the time taken for release of 10% of the added Ca^{2+}.

Significance of differences in data was based on signed ranks distribution (19).

[a] $P < .02$

[b] $P < .04$

1). The endogenous mitochondrial Ca content of Tx rats was approximately 50% that of mitochondria from paired euthyroid animals. However, as is evident from other data shown in Table 1, the endogenous mitochondrial Ca was unchanged in most of the experiments where differences in Ca^{2+} efflux were apparent and is thus unlikely to be responsible for these differences. Row B of Table 1 shows that injection of Tx rats with high doses of T3(1.0 $\mu g \cdot gm^{-1} \cdot day^{-1}$) for a period of 3 days resulted in a significant shortening of the release time. (Tx rats receiving T3 for less than 3 days showed no significant difference in Ca^{2+} efflux.) The 3-day treatment also increased α-GPDH activity but had no effect on the level of endogenous mitochondrial Ca.

Since administered T3 is known to increase the output of GH from the pituitary of the Tx rat (15), measurements were made of Ca^{2+} efflux from mitochondria of Tx rats that were given bGH (Table 1, Row C). Treatment of Tx rats with bGH (1 i.u. daily for 4 days) resulted in a shortening of the release time (stimulation of Ca^{2+} efflux) which was similar to that observed with T3 treatment. In both cases the rate at which 10% of the Ca^{2+} load was released was still considerably less than that exhibited by mitochondria from normal untreated animals, but because of day-to-day variation in Ca^{2+} efflux, which could not be explained by plasma iodothyronine levels (not shown), the significance of this finding is uncertain.

As would be expected from the previous results, mitochondria isolated from hypox rats, which lack GH and T3 as well as other hormones but which have intact parathyroid glands, released Ca^{2+} very slowly compared to normal rat liver mitochondria (Table 1, Row D). Although the endogenous mitochondrial Ca content of hypox rats was the same as that for controls, the α-GPDH activity was very low, reflecting the hypothyroid state of the hypox animals. The α-GPDH activity of the hypox rat was markedly increased by administration of 1.0 μg T3$\cdot gm^{-1}$ for 3 days (Row E). This treatment did not affect the level of endogenous mitochondrial Ca but did result in a substantial decrease in the release time to a level very similar to that observed with normal mitochondria. Injection of a much smaller amount of T3 (50 $ng \cdot gm^{-1} \cdot day^{-1}$ for 4 days)

also resulted in substantial stimulation of mitochondrial Ca^{2+} efflux, as well as α-GPDH (Row F). Administration of T3 also produced an additional acceleration of Ca^{2+} efflux in bGH-treated hypox rats (Row G).

DISCUSSION

The results of this study demonstrate that both the thyroid and growth hormone status of the rat affect the rate of Ca^{2+} efflux by isolated liver mitochondria. Despite substantial day-to-day variation in the rate of OAA-stimulated, ruthenium red insensitive Ca^{2+} efflux, results obtained with animal pairs studied on the same day were quite consistent. The results of the individual experiments performed during the study (not shown) indicated that endogenous or administered T3 or GH accelerated the regulated release of mitochondrial Ca^{2+} in 36 out of 38 pairs of rats.

The hormonal effects described here differ in direction from the rapidly-induced increase in the capacity for mitochondrial Ca^{2+} retention observed within minutes after *in vivo* administration of α-adrenergic agonists and glucagon (3). The acceleration of Ca^{2+} efflux by T3 described here required at least 3 days of exposure; the time-course of the stimulatory effect of GH has yet to be determined.

The mechanism(s) by which T3 and GH accelerate the release of mitochondrial Ca^{2+} evoked by a relatively oxidized state of the mitochondrial pyridine nucleotides is not known. One interesting possibility is that T3 (and perhaps GH) potentiate the oxidation of NADPH (16), due to inhibition of mitochondrial NADH-$NADP^+$ transhydrogenase (17). Recent evidence indicates that stimulation of mitochondria Ca^{2+} efflux by addition of OAA is due primarily to the oxidation of intramitochondrial NADPH rather than NADH (18). However, spectrophotometric determinations of the redox-state of total mitochondrial pyridine nucleotides (NAD(H) + NADP(H)) during the course of experiments such as that described by Figure 1 (8) have indicated a similar rate and extent of OAA-induced oxidation for mitochondria from normal and Tx rats (G. Fiskum, unpublished observations). Further tests will be necessary to establish whether OAA-initiated oxidation of mitochondrial NADPH *per se* is altered by

the T3 or GH status of the rat and whether these hormones affect other mitochondrial activities which could in turn influence the retention and release of mitochondrial Ca^{2+}. Studies are also currently in progress to determine if T3 and GH-induced changes in mitochondrial Ca^{2+} efflux represent physiologically-important responses to these hormones.

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