THE EFFECTS OF THYROXINE TREATMENT, IN VIVO AND IN VITRO, ON Ca^{2+} EFFLUX FROM RAT LIVER MITOCHONDRIA

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1. Introduction

We have proposed [1,2] that thyroid hormones act in vivo to stimulate mitochondrial state 4 [3] respiratory rates by inhibiting an ohmic component of the conductance of the inner mitochondrial membrane and thus elevating $\Delta\mu H^+$ and activating non-ohmic conductance pathways. Ca^{2+} recycling across the mitochondrial membrane necessitates respiratory-driven H^+ efflux to maintain the steady state and involves independent influx and efflux mechanisms [4] which exhibit ohmic [5] and non-ohmic properties [6]. Several hormones including insulin [7], glucagon [8–10] and the catecholamines [10] have been shown to influence these Ca^{2+} fluxes. Therefore, reports that Ca^{2+} efflux is enhanced by thyroid hormones added in vitro [11,12] suggested that Ca^{2+} efflux might be the non-ohmic pathway which was stimulated by our in vivo thyroxine treatment. We now report that this treatment does stimulate Ca^{2+} efflux although there are important differences between the in vitro and in vivo actions of thyroxine. However, we also show that the extra Ca^{2+} cycling does not contribute significantly to the increased respiration resulting from thyroxine treatment in vivo.

2. Methods

Male Wistar rats (250 g) were injected with either 8 mg thyroxine/kg body wt or isotonic saline, and after 24 h liver mitochondria were isolated as in [1,2]. Protein was assayed by a biuret method [13]. Mitochondrial Ca^{2+} fluxes were determined in one of two media: medium (A) contained 80 mM sucrose, 20 mM KCl, 10 mM PO_4 (as Tris–PO_4, pH 7.0), 5 mM $MgCl_2$, and 5 mM Tris–succinate; (B) was identical with (A), except for the absence of KCl and $MgCl_2$, since Mg^{2+} [14] and K^+ [15] modulate Ca^{2+} transport. Mitochondria (2 mg protein/ml) were incubated in the presence of $^{45}Ca^{2+}$ (0.6 μCi/ml) as $CaCl_2$ at either 19 or 45 natoms Ca^{2+}/mg protein. The final volume was 1.6 ml. At 0.75–4 min after the addition of the mitochondria, 0.2 ml samples were removed and spun at 10 000 × g for 0.5 min in a Beckman microfuge. The supernatant was immediately poured off and 0.05 ml was taken to 1.5 ml with scintillation fluid (Fisoflor 'mpc', Fisons, Loughborough) and counted in a Beckman LS-230. For each experiment, endogenous non-radioactive Ca^{2+} was determined [2] after adding 0.5 ml stock mitochondrial suspension to 0.5 ml 2 M perchloric acid. Full exchange ability of radioactive with non-radioactive pools of Ca^{2+} was assumed.

EGTA-induced Ca^{2+} efflux was estimated following the addition of 4 μM ruthenium red and 2 mM EGTA [16] to the medium 1 min after the mitochondria were added. Control experiments (not shown) indicated that Ca^{2+} uptake was inhibited by >98% during the time course of our incubations. The contribution of Ca^{2+} cycling to respiratory rates was estimated by assuming Ca^{2+} efflux via $Ca^{2+}/2\ H^+$ antiport and Ca^{2+} influx by Ca^{2+} uniport [6], and a succinate supported H^+:O stoichiometry of 6 [17].

Respiratory rates and ADP:O ratios were determined as in [1,2]. All incubations were stirred, and maintained at 30°C.

CCFP, ADP and ruthenium red were supplied by Sigma, Kingston Upon Thames. Ruthenium red was

Abbreviations: $\Delta\mu H^+$, mitochondrial proton electrochemical potential gradient; $\Delta\Psi$, transmembrane electrical potential; CCFP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethylenebis (oxonitrilo) tetraacetic acid

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purified [18]. $^{45}CaCl_2$ was purchased from Radiochemical Centre, Amersham.

3. Results and discussion

With mitochondria from euthyroid and thyroxine-treated rats, respiratory rates were unaffected when ruthenium red and EGTA were used to inhibit recycling of endogenous Ca^{2+} across the mitochondrial membrane (table 1). Thus our thyroxine treatment increased respiratory rates by up to 88% when recycling of endogenous Ca^{2+} was not making a significant contribution.

We also noted (table 1) that CCFP-stimulated respiratory rates were enhanced by thyroxine treatment, due presumably to a direct effect of the hormone upon the respiratory enzymes. It is not clear to what extent such a mechanism might be responsible for the stimulation of state 3 respiratory rate, which may be further improved by a hormone-mediated increase in the uptake of ADP and phosphate by the mitochondria [1,2,19]. Our measurements of Ca^{2+} efflux were made using <50 natoms Ca^{2+}/mg protein since above this level the rate of Ca^{2+} efflux increases dramatically [20]. It has been suggested [20] that at <50 natoms Ca^{2+}/protein, Ca^{2+} efflux occurred at a relatively slow rate because the matrix Ca^{2+} activity was significantly diminished as a consequence of extensive precipitation and binding of this cation. We disagree with this explanation since, as pointed out in [21], it is difficult to understand how the matrix Ca^{2+} activity could be low under these conditions since retention of the ion is almost totally dependent upon $\Delta\Psi$ and virtually all the Ca^{2+} is lost from the mitochondria within seconds of the addition of CCFP (e.g., table 2). Perhaps the increased rate of Ca^{2+} efflux following addition of >50 natoms Ca^{2+}/mg protein [20], reflects some destabilization of the mitochondrial membrane with a consequent reduction in the value of $\Delta\Psi$. Under such conditions, Ca^{2+} leaves the mitochondria via a reversal of the Ca^{2+} influx carrier [21], a process apparently insensitive to ruthenium red [22], (line 4 in, table 2). We have also observed that thyroxine treatment in vivo, appears to protect against the destabilizing effects of the higher Ca^{2+} levels [2] and to avoid these complicating effects, we have carried out our experiments with <50 natoms Ca^{2+}/mg protein.

Our data (lines 1–3 in table 2) indicate that thyroxine treatment in vivo does increase the rate of Ca^{2+} efflux from isolated mitochondria by 27–51%. The effect cannot be a consequence of the hormone uncoupling the mitochondria since the thyroxine treatment does not modify ADP:O ratios (table 1). This conclusion is further substantiated by the increases we have observed in $\Delta\mu H^+$ and ion uptake [1,2]. In the steady state, the effect of Ca^{2+} efflux (table 2) on respiratory rate (section 2) would be ⩽1 natom

Table 1
The effect of in vivo thyroxine treatment upon respiratory rates and ADP:O ratios of isolated mitochondria, and the influence of ruthenium red plus EGTA

	ADP:O	Respiratory rate (natoms O . mg protein^{-1} . min^{-1})		
		State 4	State 3	Uncoupled
Control	1.88 ± 0.2 (3)	13.5 ± 0.9 (7)	73.2 ± 3.2 (7)	59.4 ± 4.7 (4)
Control plus ruthenium red and EGTA	1.89 ± 0.17 (3)	12.6 ± 1.3 (4)	80.6 ± 11.2 (3)	n.d.
Thyroxine-treated	1.85 ± 0.16 (3)	25.4 ± 0.8 (7)	106.9 ± 9.3 (7)	98.4 ± 3.9 (4)
Thyroxine-treated plus ruthenium red and EGTA	1.85 ± 0.17 (3)	23.8 ± 1.7 (4)	123.0 ± 20.7 (3)	n.d.

Incubation conditions: medium (A) containing 2 mg mitochondrial protein/ml. ADP (0.2 μmol/mg protein) added at 2 min. Uncoupled mitochondria were those treated with 0.3 μM CCFP. Where present, 4 μM ruthenium red and 2 mM EGTA were added immediately after the mitochondria. Data include standard errors of the mean, and the number of mitochondrial preparations in parentheses; n.d., not determined

Table 2
The effect of in vivo and in vitro thyroxine treatment upon mitochondrial Ca^{2+} fluxes and respiratory rates under various conditons

Line	Medium	Ca^{2+} added (natoms/ mg protein)	Control		Thyroxine-treated	
			−RR	+RR	−RR	+RR
			ΔCa^{2+} (natoms . mg protein^{-1} . min^{-1})			
1	A	19	−0.08	−1.55	−0.07	−1.97
2	A	45	−0.18	−2.2	+0.09	−3.04
3	B	19	−0.12	−1.95	+0.08	−2.95
4	B + CCFP	19	−55	−58.2	−69.7	−79.4
5	B + Thyroxine	19	−57.2	− 4.76	n.d.	n.d.
			Respiratory rate (natoms O . mg protein^{-1} . min^{-1})			
6	A	19	16.4	16.4	26.1	31
7	A	45	17.6	19.5	35.3	33.4
8	B	19	18.8	19.9	28.4	30.8
9	B + CCFP	19	50.1	48.9	90.5	70.5
10	B + Thyroxine	19	53.0	25.6	n.d.	n.d.

Incubation conditions: medium (A) or (B) as indicated. Where present, CCFP at 0.3 μM, thyroxine at 100 μM. RR represents presence of 4 μM ruthenium red plus 2 mM EGTA. Negative ΔCa^{2+} indicates loss from mitochondria. – is inserted where rapidity of flux is such that it exceeds the ability of our technique to record it accurately. All respiratory rates are means from two mitochondrial preparations; 2 or 3 preparations were used to record Ca^{2+} fluxes; n.d., not determined

O . mg protein^{-1} . min^{-1}. Moreover, the different rates of Ca^{2+} efflux of the two populations of mitochondria account for respiratory differences of ⩽0.3 natoms O . mg protein^{-1} . min^{-1}, i.e., 3% of the respiratory enhancement due to thyroxine treatment (lines 3,8 in table 2). It is unlikely that we underestimated the extent of Ca^{2+} efflux, because of any inhibition by ruthenium red, since the latter did not inhibit respiratory rate (lines 6–8 in table 2).

Our data may be compared with those in [12]; following the accumulation by heart mitochondria of 20 natoms Ca/mg protein in the absence of Mg^{2+}, ruthenium red and ~50 nmol thyroxine/mg protein were added. The latter induced a >10-fold stimulation in the rate of Ca^{2+} efflux and a similar phenomenon was observed in liver mitochondria [11]. We have confirmed this in vitro action of thyroxine (compare lines 3 and 5 in table 2) but the interpretation of these results is likely to be complicated. For instance, in the absence of ruthenium red, the thyroxine-induced increase in respiratory rate (line 10) was not further enhanced by CCFP (not shown). Moreover, the rate of Ca^{2+} efflux from mitochondria treated with thyroxine in vitro (line 5) was similar to that from uncoupled mitochondria (line 4). Thus thyroxine in vitro may uncouple the mitochondria [23] inducing a reversal of the Ca^{2+} influx mechanism [21]. Alternatively, if the hormone activates the independent Ca^{2+} efflux carrier, subsequent Ca^{2+} recycling may uncouple the organelles. In any case, these events differ from the effects of thyroxine treatment in vivo (see above). Note also that ruthenium red largely reverses or inhibits the increases in rates of Ca^{2+} efflux and respiration caused by thyroxine in vitro (lines 5 and 10). In conclusion, thyroxine treatment in vivo stimulates Ca^{2+} efflux in contrast to the inhibitory effect of glucagon and catecholamines [10] on this process. However, the thyroid hormone effects on Ca^{2+} efflux do not appear to contribute significantly to the enhancement of respiratory rate by the hormone.

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