Effect of Calcium Ion on Triiodothyronine Binding to Kidney Outer Mitochondrial Membrane *in vitro*

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Abstract

The effect of calcium ion on 3,5,3'-triiodothyronine (T_3) binding to rat kidney outer mitochondrial membranes was examined *in vitro*. The outer mitochondrial membranes were prepared by using a discontinuous sucrose density gradient centrifugation. The membrane fraction, which is enriched with monoamine oxidase activity, contained specific binding sites for T_3 . Scatchard analysis of T_3 binding to outer mitochondrial membranes gave an association constant (K_a) of $0.53 \times 10^{10} M^{-1}$. The binding of [^{125}I]- T_3 to the membranes was inhibited by the addition of $CaCl_2$ ($0.25 \times 10^{-4} - 2.5 \times 10^{-3} M$). 50% inhibition was obtained by $0.75 \times 10^{-4} M$ $CaCl_2$ in the presence of 0.1 mM EGTA. When outer mitochondrial membranes were solubilized with Triton X-100, four main T_3 binding activities were isolated by a gel filtration study. On the other hand, the binding of [^{125}I]- T_3 to the solubilized T_3 receptors derived from outer mitochondrial membranes was not strongly inhibited by calcium.

When outer mitochondrial membranes were preincubated in the presence of 1 mM calcium, the number of T_3 binding sites in the membranes was decreased, and this was associated with an increase in the number of T_3 binding sites in the supernatants of the incubation mixture. Scatchard analysis showed that the number of T_3 binding sites in the membranes is decreased by calcium ion without any change in the association constant. In studies with gel filtration of receptors which are released by Ca^{2+} from outer mitochondrial membranes, three main T_3 binding activities were isolated. Mg^{2+} , Mn^{2+} , Zn^{2+} and Cu^{2+} did not affect T_3 binding to outer mitochondrial membranes.

The results indicate that calcium ion regulates T_3 binding to the outer mitochondrial membrane through the release of T_3 receptors from the membranes.

It is well established that mitochondria play an important role in storing large amounts of calcium. The calcium concentration in the extramitochondrial space is strictly regulated by the mitochondrial cal-

cium transport system (Carafoli and Crompton, 1978). Several hormones modify the calcium transport system in mitochondria (Rasmussen and Gustin, 1978). Thyroid hormone stimulates calcium release from mitochondria (Shears and Bronk, 1981, Harris, 1979). The mechanism of the stimula-

tion of calcium release is known to be a result of thyroid hormone-induced augmentation of mitochondrial permeability to calcium (Harris, 1979, Hullert *et al.*, 1976, Statav *et al.*, 1976). The changes in the calcium concentration in the extramitochondrial space may induce the modification of many aspects of metabolism in this space. Calcium-dependent proteinase activity (Guroff, 1964, Murachi *et al.*, 1980, Murachi *et al.*, 1981), calcium binding protein (calmodulin), and several calcium-requiring enzyme activities may be modified by the changes in calcium concentration. An increase in the calcium concentration in this space, which is induced by thyroid hormone, may also induce modifications of these enzymes.

We have previously reported that outer and inner mitochondrial membranes have specific binding sites for 3,5,3'-triiodothyronine (T_3) (Hashizume & Ichikawa, 1982). And we suggested that the binding is regulated by calcium ion *in vitro*. However, the mechanism of inhibition of T_3 binding to the membranes is not yet known. An increase in calcium concentration may activate the calcium-activated proteinase activity and may induce release of T_3 receptors, or inactivation of receptors.

In the present study, we examined whether or not calcium ion has a regulatory effect on T_3 binding to outer mitochondrial membranes *in vitro*. Our results indicate that calcium ion decreases the number of T_3 binding sites in outer mitochondrial membranes, and that is accompanied by the release of T_3 receptors from outer mitochondrial membranes.

Materials and Methods

Preparation of mitochondrial membranes

Mitochondria were prepared from male Wistar rat kidney by the method of Mela and Seits (1979) with minor modifications. All procedures were done in the presence of 1.0 mM EGTA and 1.0 mM dithiothreitol (DTT) (Sigma

Chemical Co.). The outer and inner membranes were prepared by the method of Comte and Gautheron (1979) by using discontinuous sucrose density gradient centrifugation. As a marker enzyme located on the outer mitochondrial membranes, monoamine oxidase (MAO) activity was measured by the method of Wurtman and Axelrod (1963) using [14 C]-tryptamine bisuccinate (SA; 30 mCi/mmol) (New England Nuclear, Boston, MA, U.S.A) as substrate (Ichikawa *et al.* 1982).

Solubilization of mitochondrial membranes

Solubilization of mitochondrial membranes was done as follows. The membrane fraction (5–10 mg protein) was incubated in 50 mM sodium phosphate buffer pH 7.5, containing 0.25% Triton X-100, 1.0 mM EGTA, 1 mM DTT and 5% glycerol for 1 hr at 4°C. After incubation, the mixture was centrifuged at 105,000×*g* for 1 hr at 0°C. The supernatant was used as a fraction contained soluble T_3 receptors, and the pellet suspended with the same volume of the buffer was used as the residual membrane fraction. To remove Triton X-100 from these fractions each fraction was passed through a small column of Amberlite XAD-2 (Sigma) which had been equilibrated with the same buffer that did not contain Triton X-100.

Receptor release from mitochondrial membranes

The effect of Ca^{2+} on the release of T_3 receptors from outer mitochondrial membrane was examined as follows. The membrane fraction (5–10 mg of protein) was incubated with $CaCl_2$ in 50 mM phosphate buffer pH 7.5, containing 1.0 mM DTT for 1 hr at 34°C. In the control, $CaCl_2$ was omitted and 1.0 mM EGTA was added to the incubation medium. After incubation the mixture was centrifuged at 105,000×*g* for 1 hr at 0°C. The precipitate was suspended with the same volume of the buffer which did not contain $CaCl_2$ and was used as the residual membrane fraction. The supernatant contained released T_3 receptors. Both preparations were dialyzed against 50 mM phosphate buffer pH 7.5, containing 1.0 mM EGTA and 1.0 mM DTT overnight, and the T_3 binding activity in these fractions was measured.

T_3 binding assay

Binding assay was carried out by the method of Sterling and Milch (1975). The reaction mixture contained 0.1 mM EGTA, 1.0 mM DTT and

a trace amount of [^{125}I]- T_3 (10,000–20,000 cpm) (SA; 150 $\mu\text{Ci}/\mu\text{g}$) (New England Nuclear) in a total volume of 350 μl of 50 mM sodium phosphate buffer pH 7.5. The reaction was started by adding 100 μl aliquotes of either mitochondrial membrane fraction or soluble (or released) receptor fraction, and incubation was performed in the presence or absence of unlabeled T_3 (10^{-6}M) (Sigma) for 1 hr at 30°C . The reaction was terminated by cooling the mixture in an ice bath. In the case of T_3 binding to the membranes, the membrane-bound hormone was separated by centrifugation of the incubation mixture at $105,000\times g$ for 30 min. at 0°C . In the case of T_3 binding to the solubilized or released T_3 receptors, the free hormone was separated from the bound hormone with Dextran-coated charcoal (Norit "A" Dextran 50,000–90,000 molecular weight, both 10 g/l). Specific binding of [^{125}I]- T_3 was calculated by subtraction of the [^{125}I]- T_3 bound in the presence of 10^{-6}M of unlabeled T_3 (Hashizume and Ichikawa, 1982).

Gel filtration of T_3 receptors was carried out on a column of Sephadex G-100 (Pharmacia), $60\times 2\text{ cm}$, and the receptors were eluted with 50 mM phosphate buffer pH 7.5, containing 0.1 mM EGTA, 1.0 mM DTT and 5% glycerol. The protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) (Sigma) as a standard.

Results

Localization of T_3 receptors in rat kidney mitochondrial membranes

As we have previously demonstrated, the MAO-enriched fraction which is considered to be the outer mitochondrial membrane fraction contained high T_3 binding activity (Hashizume and Ichikawa, 1982). Cytosol, microsomal and inner mitochondrial membrane fractions also contained T_3 binding activity, but specific activity for T_3 binding to these fractions was lower than that of outer mitochondrial membrane fraction (Table 1). [^{125}I]- T_3 binding to outer mitochondrial membrane was displaced by unlabeled T_3 in a concentration dependent manner, and 50% displacement was obtained by $0.7\times 10^{-9}\text{M}$ of unlabeled T_3 . The association constant (K_a) of binding to outer mitochondrial membranes was $0.53\times 10^{10}\text{M}^{-1}$ (Hashizume and Ichikawa, 1982).

The effect of calcium ion on T_3 binding to outer mitochondrial membranes

[^{125}I]- T_3 binding to outer mitochondrial membranes was reduced by the addition of calcium ion *in vitro*. Fig. 1 shows a typical concentration curve for the inhibition of binding of [^{125}I]- T_3 to outer mitochondrial

Table 1. Localization of T_3 binding activities in rat kidney mitochondrial membranes.

Fraction	[^{125}I]- T_3 binding (cpm/100 μg of protein)	MAO activity ([^{14}C]-tryptamine oxidized) (nmoles/ μg protein $\cdot$ 20 min)
Exp. 1		
Cytosol	750	2.62
Microsomal	980	1.54
Inner mitochondrial membrane	2180	1.94
Outer mitochondrial membrane	3620	28.44
Exp. 2		
Cytosol	840	2.21
Microsomal	689	1.04
Inner mitochondrial membrane	2620	2.01
Outer mitochondrial membrane	4400	29.36

Each value is the mean of triplicate determinations.

Fig. 1. Effect of calcium ion on T₃ binding to inner or outer mitochondrial membranes.

Mitochondrial membranes were prepared from rat kidney mitochondria as mentioned in the Materials and Methods. The binding assay was carried out in the presence of 100 μ M EGTA and various concentrations of Ca²⁺. 10⁵ cpm of [¹²⁵I]-T₃ were added in each reaction. The specific binding of T₃ is shown. Each result is the mean of triplicate determinations.

Table 2. Effect of cations on T₃ binding to outer mitochondrial membranes.

Cations	% of binding in the absence of cation		
	Concentration (×10 ⁻⁴ M)		
	0.5	1.0	2.5
Ca ²⁺	41.6	29.7	20.8
Mg ²⁺	88.1	78.7	72.8
Mn ²⁺	88.6	72.1	66.8
Zn ²⁺	112.1	126.6	72.6
Cu ²⁺	149.2	97.3	57.7

Each value is the mean of triplicate determinations.

Table 3. [¹²⁵I]-T₃ binding to solubilized fraction of outer mitochondrial membranes.

Fraction	[¹²⁵ I]-T ₃ binding (cpm/100 μ g of protein)
Outer membrane	
Outer membrane before solubilization	2830
Residual outer membrane	130
Soluble fraction	26860

Residual outer membrane and soluble fraction were prepared by using Triton X-100 as mentioned in the Materials and Methods. Soluble fraction indicates the 105,000×g supernatant after treatment of the outer membrane with Triton X-100. Each value is the mean of triplicate determinations.

Fig. 2. Effect of calcium ion on T₃ binding to solubilized fraction prepared from outer mitochondrial membranes.

Binding reaction contained various concentrations of Ca²⁺ and solubilized protein (100 μ g/tube). The reactions were performed in the presence or absence of 10⁻⁶M unlabeled T₃. The specific binding of T₃ was illustrated. Each value is the mean of triplicate determinations.

membrane preparation by calcium ion. Calcium ion in the concentration from 0.25×10^{-4} to $2.5 \times 10^{-3}\text{M}$ strongly inhibited the $[^{125}\text{I}]\text{-T}_3$ binding. The binding of $[^{125}\text{I}]\text{-T}_3$ to inner membrane was also inhibited by calcium ion. Other divalent cations, such as Mg^{2+} , Mn^{2+} , Zn^{2+} and Cu^{2+} , in their lower concentrations, did not inhibit $[^{125}\text{I}]\text{-T}_3$ binding to outer mitochondrial membranes. Higher concentrations of these ions, however, inhibited $[^{125}\text{I}]\text{-T}_3$ binding to the membrane (Table 2).

The effect of calcium ion on T_3 binding to soluble T_3 receptors prepared from outer mitochondrial membranes

Soluble fraction of Triton X-100-treated mitochondrial membranes contained binding sites for T_3 . Specific activity for T_3 binding was increased 9.4 fold by solubilization (Table 3). Calcium ion was tested for its ability to inhibit $[^{125}\text{I}]\text{-T}_3$ binding to the receptors in this fraction. Even 1 mM calcium ion did not inhibit T_3 binding to solubilized receptors (Fig. 2). In studies with gel filtration of solubilized receptors, four main T_3 binding activities were isolated (Hashizume and Ichikawa, 1982). Calcium

ion was again tested for its ability to inhibit T_3 binding to each receptor. Calcium ion slightly inhibited $[^{125}\text{I}]\text{-T}_3$ binding to receptors present in B fraction in Fig. 3. When C and D fractions, obtained by the gel filtration study shown in Fig. 3, were used as receptor fractions, calcium ion did not inhibit $[^{125}\text{I}]\text{-T}_3$ binding to these receptors. Calcium ion, in these cases, enhanced binding of T_3 to these receptors. Binding of T_3 to receptor C was enhanced by lower calcium concentrations (0.125×10^{-3} – $0.25 \times 10^{-3}\text{M}$) and that to fraction D was enhanced by higher concentrations of calcium ion (more than $0.5 \times 10^{-3}\text{M}$), as shown in Fig. 4.

Ca^{2+} -induced T_3 receptor release from outer mitochondrial membranes

The above results suggest that Ca^{2+} does not directly inhibit T_3 binding to receptor molecules and that Ca^{2+} may interact with outer mitochondrial membrane and may result in a decrease in binding activity in the membrane. In order to investigate the mechanism of Ca^{2+} -induced reduction of T_3 binding activity in the membrane, Ca^{2+} -pretreated membranes were tested for their

Fig. 3. Gel chromatography profile of soluble T_3 receptors.

The outer mitochondrial membranes were solubilized by 0.25% Triton X-100. Solubilized membrane fraction was applied to a Sephadex G-100 column and the receptors were eluted. The binding activity in each fraction was assayed in the absence or presence of unlabeled T_3 . The elution positions of BSA and cytochrome c (Cyt. C) are indicated.

ability to bind T_3 .

During the incubation of outer mitochondrial membranes with 1 mM Ca^{2+} , the binding activity for T_3 in the membranes decreased. T_3 binding activity in the supernatant of the incubation mixture of the membranes with calcium ion, on the contrary, was increased in a time dependent manner (Fig. 5). Fig. 6 show the T_3 binding activity in the Ca^{2+} -pretreated outer mitochondrial membranes and that in the supernatant of the incubation mixture. Scatchard analysis indicated a decrease in the number of T_3 binding sites on outer mito-

chondrial membranes without any change in the association constant after pretreatment of the membranes with Ca^{2+} (Fig. 7). In studies with gel filtration on a Sephadex G-100 column, three main T_3 binding activities were isolated in the supernatant of the incubation mixture (Fig. 8). Pretreatment of outer mitochondrial membranes with Mg^{2+} or Mn^{2+} did not produce any soluble binding activity for T_3 .

Fig. 4. Effect of Ca^{2+} on T_3 binding to solubilized T_3 receptors.

Solubilized T_3 receptor fractions were obtained from the experiment shown in Fig. 3. Fraction B, C and D were studied. Binding reactions contained various concentrations of Ca^{2+} and solubilized protein (16 μ g/tube). The specific binding of T_3 is shown. Each value is the mean of triplicate determinations.

Fig. 5. Effect of pretreatment of outer mitochondrial membranes with Ca^{2+} on the release of T_3 receptors.

Outer mitochondrial membranes (7.5 mg protein) were incubated in the presence of 1 mM Ca^{2+} at 34°C for appropriate incubation time. After the incubation, the mixture was centrifuged at 105,000 $\times g$ for 1 hr at 0°C. The pellet was resuspended with the same volume of Ca^{2+} -omitted buffer containing 1 mM EGTA, and was used as a residual membrane fraction. After dialysis of both the residual membrane fraction and supernatant against Ca^{2+} -omitted buffer containing 1 m EGAT, $[^{125}I]-T_3$ binding activity in both the fractions was determined. The reaction was performed by adding 100 μ l of aliquotes of either membrane fraction (—○—) or soluble fraction (—●—). Each value is the mean of triplicate determinations.

Fig. 6. Effect of pretreatment of outer mitochondrial membranes with Ca^{2+} on the release of T_3 receptors.

Outer mitochondrial membranes (7.5 mg protein) were incubated in the presence or absence of 1 mM Ca^{2+} for 1 hr at 34°C . In control group, 1 mM EGTA was added to Ca^{2+} -omitted medium. After the incubation, the soluble and residual membrane fractions were separated by centrifugation at $105,000 \times g$ for 1 hr at 0°C . Binding activity in the residual membrane fraction (left panel) and in the soluble fraction (right panel) was determined after dialysis of these fractions against Ca^{2+} -omitted buffer containing 1 mM EGTA. Buffer-treated indicates the control group. Each value is the mean of duplicate determinations.

Fig. 7. Scatchard analyses of the binding of $[^{125}\text{I}]\text{-T}_3$ by buffer-treated (—●—) and by Ca^{2+} -treated (—○—) outer mitochondrial membranes. Both the membrane fractions were prepared as described in Fig. 6. The T_3 binding assay was performed as mentioned in Materials and Methods. Each membrane fraction ($50 \mu\text{g}$ of protein) was incubated with various concentrations of $[^{125}\text{I}]\text{-T}_3$ in the presence or absence of unlabeled T_3 (10^{-6}M) for 1 hr at 30°C . The specific binding of $[^{125}\text{I}]\text{-T}_3$ was determined. Each value is the mean of triplicate determinations.

Fig. 8. Gel filtration profile of soluble T_3 receptors.

The soluble fractions were prepared by pretreatment of outer mitochondrial membranes with Ca^{2+} containing buffer or Ca^{2+} -omitted buffer as described in Fig. 6. Soluble fractions were applied to a Sephadex G-100 column and the receptors were eluted with the buffer which did not contain Ca^{2+} . The T_3 binding activity in each fraction was assayed in the presence or absence of unlabeled T_3 ($10^{-6}M$). The specific binding is shown. —●—; binding activity in soluble fraction obtained from the control group which was prepared by treatment of the membrane with buffer containing 1 mM EGTA. —○—; binding activity in soluble fraction obtained from Ca^{2+} -treated outer mitochondrial membranes. Soluble fraction obtained from Mg^{2+} - or Mn^{2+} -treated membranes gave a similar result to that of the control group. The elution positions of BSA and cytochrome *c* (Cyt. C) are shown. V.V. indicates the position of the void volume of the column.

Discussion

In the present study we have demonstrated that calcium ion plays an important role in the regulation of binding of T_3 to outer mitochondrial membranes. The mechanism of this regulation is an inhibition of calcium on T_3 binding to the membranes. We also demonstrated that the inhibitory effect of calcium ion is mediated by the release of T_3 receptors from outer mitochondrial membranes.

It is known that Ca^{2+} is released from mitochondria by thyroid hormones (Harris, 1979, Shears and Bronk, 1981). The increase in the calcium concentration in the

extramitochondrial space may stimulate certain reactions which require Ca^{2+} . A thyroid hormone-induced increase in the calcium concentration in this space may regulate thyroid hormone action at sites subsequent to T_3 binding to outer mitochondrial membranes. As is shown in the present study, calcium ion strongly inhibited T_3 binding to outer mitochondrial membranes, suggesting that calcium ion, which is released from mitochondria by thyroid hormone stimulation, plays a role in the regulation of T_3 binding to the membranes. Possibly this mechanism is a feedback regulation of thyroid hormone action in the intracellular space. Other divalent cations also inhibited T_3 binding in higher concentrations, but not

in their physiological concentrations, suggesting that calcium ion is a specific regulator for T_3 binding.

Ca^{2+} did not inhibit T_3 binding to soluble T_3 receptors derived from outer mitochondrial membranes, suggesting that calcium ion does not interact directly with T_3 receptor molecules. However, we obtained results which indicate Ca^{2+} enhances T_3 binding to the isolated soluble T_3 receptors. We cannot explain whether or not these enhancements are physiological.

The decrease in the number of T_3 binding sites in outer mitochondrial membranes and the appearance of soluble T_3 binding activities in the supernatant after treatment of the membranes with Ca^{2+} , indicated that Ca^{2+} inhibition of T_3 binding to the membranes occurs through the release of T_3 receptors from outer mitochondrial membranes. Recently, calcium-activated proteinases in several tissues have been isolated (Guroff, 1964, Murachi *et al.*, 1980, Murachi *et al.*, 1981). Although it is not known whether Ca^{2+} -activated proteinases interact with outer mitochondrial membranes, it is possible that a thyroid hormone-induced increase in calcium concentration in the extramitochondrial space activates these proteinases and that results in structural changes in the outer mitochondrial membranes or degradation of some molecules, including T_3 receptors, in the membranes.

In the gel filtration study, we obtained different T_3 binding activity profiles in soluble fraction for Triton X-100 and Ca^{2+} -treated. Probably these treatments produce different molecular sized soluble T_3 receptors. Furthermore, although T_3 binding to isolated Triton X-100-solubilized- T_3 receptors was modified by Ca^{2+} , that to the isolated receptors released by Ca^{2+} was not affected by Ca^{2+} (Data not shown). These results suggest that not only the size of T_3 receptors but also T_3 binding characteristics of T_3 receptors released by Ca^{2+} are different from those solubilized by Triton X-100.

Several workers could not detect the thyroid hormone receptors in outer mitochondrial membranes (Sterling and Milch, 1975, Tata *et al.*, 1962 *a* and *b*). Since calcium ion can release T_3 receptors from outer mitochondrial membranes, it might be easy to lose the receptors from the membranes in the presence of calcium ion. In our experiments 1 mM EGTA was employed during preparation of membranes, and we could detect T_3 receptors in the MAO-enriched fraction which is considered to be the outer mitochondrial membrane fraction.

In this report we did not use the agents which inhibit calcium efflux from mitochondria. And it is necessary to study the effect of these agents on T_3 binding to mitochondria. While the role of calcium ion in T_3 binding to outer mitochondrial membrane is no doubt important, the relationship between Ca^{2+} , which is released from mitochondria by thyroid hormone, and T_3 binding to the membrane remains to be elucidated.

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