

Thyroid Hormones and Active Calcium Transport of Inside-Out Red Cell Membrane Vesicles

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Thyroid hormones may influence the active transport of Ca^{2+} across the cell membrane. To test the physiologic relevance of this mechanism, we used inside-out human red cell membrane vesicles as a model of the cell membrane Ca^{2+} pump. We monitored by spectrophotometric methods the kinetics of the uptake of Ca^{2+} in the presence of 10^{-5} – 10^{-10} M thyroid hormones or their analogues. Vesicles freed of calmodulin and protein inhibitor(s) of the Ca^{2+} pump were also obtained. The results are as follows: (1) Thyroxine inhibits the active Ca^{2+} uptake; (2) this effect antagonizes that of soluble calmodulin; and (3) triiodothyronine and other analogues of the thyroid hormones are less active than thyroxine. We conclude that the thyroid hormones may influence cell Ca^{2+} homeostasis by direct action on the Ca^{2+} pump. © 1992 Academic Press, Inc.

The mechanism underlying the regulation of the cell activities by thyroid hormones¹ involves the nuclear components of the cell (1). In addition to this mechanism, it was found that TH affect the activity of Ca^{2+} -ATPase or the response of Ca^{2+} -ATPase to CaM (2,3). It was thus proposed that the action of TH is also exerted through modulation of the homeostasis of Ca^{2+} . However, Ca^{2+} -ATPase is only one of the components of the Ca^{2+} pump, which is influenced by several interacting factors including the microenvironment of the enzyme, CaM, the protein inhibitors of the Ca^{2+} pump and other intracellular factors (4). Therefore, the physiologic relevance of that observation is questionable.

We investigated the effect of TH on the active Ca^{2+} transport by IORCMV to test whether the observed stimulation on Ca^{2+} -ATPase persists in a physiologic model. The IORCMV (5) are particularly suitable for this purpose because the intracellular structures are ruled out. The reversed orientation of the membrane

¹ Abbreviations used: CaM, calmodulin; IORCMV, inside-out red cell membrane vesicles; RBC, red blood cell; TH, thyroid hormones; T_4 , 3,3',5,5'-L-thyroxine; T_3 , 3,3',5-triiodo-L-thyronine; DT_3 , 3,3',5-triiodo-D-thyronine; rT_3 , 3,3',5'-triiodo-L-thyronine; Triac, 3,3',5-triiodothyroacetic acid.

exposes its inner face to the experimental conditions eliminating interferences by cell surface receptors and internalization processes. In addition, membrane-bound CaM and protein inhibitor(s) of the Ca^{2+} pump were selectively removed to identify the role of these factors. The specificity of the effect of TH was assessed using structural analogues.

MATERIALS AND METHODS

Reagents. All reagents were provided from Sigma Chemicals (St. Louis, MO) and were analytical grade. Arsenazo III was freed of Ca^{2+} ions by passage through a Chelex-100 resin (Bio-Rad Laboratories).

Inside-out red cell membrane vesicles. Fresh heparinized blood samples were obtained from euthyroid and healthy female volunteers (age 25 ± 4 years, means \pm SE, range 21–35 years) as part of a study into the pathogenesis of disturbances of mineral metabolism which frequently affect the female population. The IORCMV were prepared as previously described (6) washing the RBC with 154 mM NaCl, hemolyzing them with 5 mM phosphate buffer at pH 8, and repeatedly washing the ghosts with the same buffer. To obtain vesicles where endogenous CaM and the inhibitors of the Ca^{2+} pump are quantitatively removed, part of the ghosts were washed three times with 5 mM phosphate at pH 8 and 1 mM EDTA (7). EDTA was removed by further washing IORCMVs in EDTA-free buffer three times. Ghosts were finally incubated overnight at 4°C in a 0.5 mM phosphate at pH 8.

Vesiculation was initiated by passage through a 27 gauge \times $\frac{3}{4}$ in. needle, and the percentage of inside-out vesicles was estimated by the acetylcholinesterase accessibility assay (5). The total protein concentration of the IORCMV suspension was measured with the bicinchoninic acid assay (Pierce Chemical).

The active calcium uptake. The calcium uptake by IORCMV was monitored at 37°C by the absorbance change recorded by a dual-wavelength spectrophotometer DW2A (American Instrument Co., Silver Spring, MD) operating at 675–685 nm to offset the interferences due to the turbidity of the suspension, Mg^{2+} , and pH. The active Ca^{2+} uptake was initiated adding 0.5 nM ATP to the incubation medium containing the vesicles (3–4 mg/ml total protein), 0.02 mM CaCl_2 , 1 mM MgCl_2 , 0.1 M KCl, 5 mM sodium azide, and 0.1 mM Arsenazo III. The maximally activated calcium uptake was obtained adding 250 IU CaM to the mixture. Data are expressed as micromoles of Ca^{2+} uptaken per minute per milligram of protein and are corrected for the actual percentage of inside-out vesicles.

Thyroid hormones. TH (Sigma) were dissolved in 0.5 mM phosphate, pH 8, at 25°C , titrated to pH 7.5 with NaOH, and were kept at 0°C for no longer than 4 h. The kinetics of Ca^{2+} uptake were measured using various amounts of TH in the same volume (10 μl). TH were added to the cuvette during the linear portion of the active Ca^{2+} uptake.

Statistics. All data are expressed as means \pm SE and compared using the Student's *t* test for unpaired observations. When the inhibition by TH and their analogues was considered, the paired Student's *t* test was used.

TABLE 1
Effect of T_4 on the Ca^{2+} Uptake ($\mu\text{mol } Ca^{2+}/\text{min}/\text{mg}$ protein) by Untreated and EDTA-Treated IORCMV

IORCMV	CaM 250 IU/ml	n	10^{-5} M T_4		P*
			Before	After	
Untreated	No	6	1.6 ± 0.3	1.5 ± 0.3	NS
	Yes	15	2.3 ± 0.1	1.8 ± 0.1	<0.0005
EDTA-treated ^a	No	8	0.9 ± 0.2	0.9 ± 0.2	NS
	Yes	12	2.4 ± 0.3	2.0 ± 0.3	<0.0005

Note. Data are means \pm SE.

^a Endogenous CaM and inhibitors removed.

* The significance level refers to the difference between the Ca^{2+} uptake before and after addition of T_4 (Student's *t* test for paired data).

RESULTS

The IORCMV preparations tested in this study were stable and linear during the maximally activated Ca^{2+} uptake for at least 5 min (not shown). No Ca^{2+} chelation by TH was evident from lack of absorbance changes on addition of TH to the medium.

In the absence of soluble CaM, the addition of 10^{-5} M T_4 did not affect the active Ca^{2+} uptake of IORCMV (Table 1). In contrast, T_4 inhibited the active Ca^{2+} uptake of untreated IORCMV by $21 \pm 2\%$ in the presence of excess soluble CaM. When the IORCMV were treated with EDTA to remove membrane-bound CaM and the inhibitor(s) of the Ca^{2+} pump, the uptake of Ca^{2+} decreased, but the maximally activated uptake was independent of the treatment, as expected (6). Under these conditions, the inhibition by T_4 was $17 \pm 3\%$ in the presence of soluble CaM.

The magnitude of the inhibition exerted by T_4 on the maximally activated Ca^{2+} uptake was dose dependent in the 10^{-10} – 10^{-5} M T_4 range (Fig. 1). The inhibition was unaffected by pretreatment of the ghosts with EDTA.

The effect of triiodothyronine and other analogues (rT_3 , DT_3 , and Triac) on the maximally activated Ca^{2+} uptake was tested at 10^{-5} M TH (Table 2).

DISCUSSION

In the IORCMV model, T_4 inhibited the Ca^{2+} pump in the presence of excess soluble CaM in the medium. No effect of T_4 was observed in the absence of soluble CaM. This feature was not dependent on the presence of membrane-bound CaM and of the protein inhibitor(s) of the Ca^{2+} pump. The effect of T_4 was dose dependent without apparent saturation of the response in the 10^{-5} – 10^{-10} M T_4 range. The analogues of TH inhibited the Ca^{2+} uptake but to a lower extent than T_4 .

Due to the peculiarities of the IORCMV model, the effect of T_4 on the maximally activated Ca^{2+} uptake appears associated to factors present on the cell membrane. Cell surface receptors and carriers are not involved, but the presence

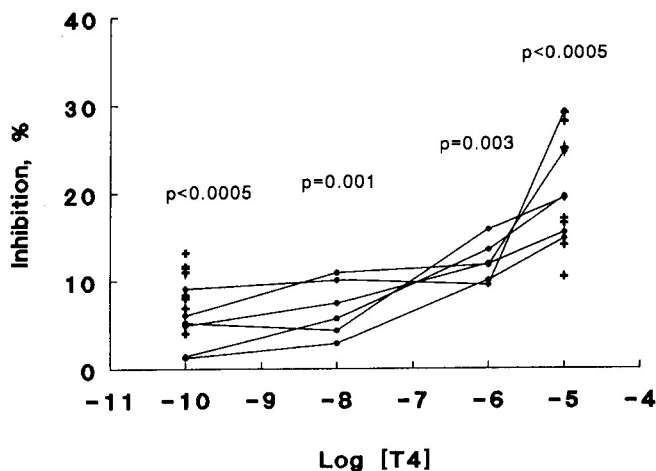


FIG. 1. Inhibition of the maximally activated Ca^{2+} uptake at varying concentrations of T_4 in untreated IORCMV ($20 \mu\text{M}$ Ca^{2+} , $500 \mu\text{M}$ ATP, and 250 IU/ml CaM). All data obtained under these conditions are shown and were used for the Student's *t* test for paired observations. Some samples, analyzed at different concentrations of T_4 , are indicated by solid lines.

of soluble CaM is critical. Membrane-bound CaM and protein inhibitor(s) are not relevant.

Previous work on the effect of TH on the active Ca^{2+} transport in the RBC membrane concerned the hydrolytic activity of Ca^{2+} -ATPase (2,3,8). Incubation (60–120 min at 37°C) of RBC ghosts with T_4 or T_3 stimulated such activity (2), likely through interaction with CaM (8). These results are in contrast with the loss of any effect by T_4 following maximal activation of Ca^{2+} -ATPase by CaM(3). These authors suggest an indirect effect of T_4 on RBC membrane stability, lipid microdomain or CaM binding.

The inhibition of the Ca^{2+} pump activity by T_4 reported here is in apparent

TABLE 2
Effect of 10^{-5} M T_4 and Analogues on the Active CaM-Activated Transport of Ca^{2+} in the Inside-out Red Cell Membrane Vesicles

	<i>n</i>	Basal	CaM-activated	After addition of TH analogue	% Inhibition	<i>P</i>
T_4	15	1.0 ± 0.1	2.3 ± 0.1	1.8 ± 0.1	21 ± 2	<0.0005
T_3	10	1.3 ± 0.2	3.0 ± 0.4	2.8 ± 0.4	8 ± 2	0.002
r T_3	6	1.6 ± 0.3	3.3 ± 0.4	3.1 ± 0.4	7 ± 2	0.048
DT $_3$	7	1.4 ± 0.2	3.0 ± 0.3	2.6 ± 0.3	12 ± 2	0.008
Triac	8	1.4 ± 0.2	2.9 ± 0.2	2.8 ± 0.2	3 ± 3	NS

Note. T_4 = 3,3',5,5'-L-thyroxine; T_3 = 3,5,3'-triiodo-L-thyronine, r T_3 , 3,3',5'-triiodo-L-thyronine; DT $_3$, 3,3',5-triiodo-D-thyronine; Triac, 3,3',5-triiodothyroacetic acid. Ca^{2+} uptake expressed as $\mu\text{mol Ca}^{2+}/\text{min}/\text{mg}$ protein.

contrast with the above reports. If differences in the lipid composition of the plasma membrane and sex/age of the donors are ruled out (9), the importance of the experimental model employed becomes critical. We believe that the IORCMV model is better suitable than purified Ca^{2+} -ATPase to examine the reactivity of the Ca^{2+} pump toward perturbators. In fact, the IORCMV model integrates several components of the Ca^{2+} pump system, i.e., the hydrolytic and transport functions, the lipid microdomain of the enzyme, endogenous CaM, and the inhibitors of Ca^{2+} -ATPase. In contrast, the Ca^{2+} -ATPase model is based on the enzyme hydrolytic activity, which is one only component of the Ca^{2+} pump assembly. It was already suggested that Ca^{2+} -ATPase and the Ca^{2+} pump functions could be dissociated in the presence of TH (10), and that the response of the enzymatic system to the action of T_4 could be conditioned by the lipid microdomain of the enzyme (9,10).

The dependency on the concentration was tested in the 10^{-10} – 10^{-5} M T_4 range. Although the physiologic relevance of the latter concentration is questionable, it is worth note that the carrier-mediated membrane transport system can concentrate TH into the cell up to 74 times the concentration found in plasma when rat liver cells are incubated with physiologic concentrations of T_3 for 60 s (11). Molecular alterations of T_4 reduced the inhibition of the Ca^{2+} pump indicating that this effect is highly specific for T_4 .

In conclusion, this study indicates that T_4 exerts an inhibition on the RBC membrane Ca^{2+} pump similar to the action of TH on oxidative phosphorylation in liver mitochondria which is not mediated by interaction with the nucleus (12). Interaction of soluble CaM to Ca^{2+} -ATPase is critical in this mechanism. An increasing amount of evidence indicates that the characteristics of RBC membrane Ca^{2+} -ATPase are similar to those of other systems such as osteoblasts (13), adipocytes (14), and renal tubular cells (15). Thus, the mechanism described here may be extended to other cell types as well, as already suggested (10).

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