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Effect of Extracellular Sodium on Thyroid Hormone Uptake by Mouse Thymocytes*

M. CENTANNI, A. SAPONE, A. TAGLIENTI, AND M. ANDREOLI

Second Chair of Endocrinology, Department of Experimental Medicine, University La Sapienza, Rome, Italy

ABSTRACT. In mouse thymocytes, a stereospecific saturable energy-dependent and ouabain-inhibitable system facilitates T_3, but not T_4, entry. We studied here the effect of sodium depletion on cellular uptake of thyroid hormones by mouse thymocytes. Time-course experiments indicated that extracellular sodium depletion reduced $[^{125}I]T_3$ uptake at each time studied. At equilibrium, the removal of extracellular sodium and its substitution with isoosmotic choline decreased saturable $[^{125}I]T_3$ uptake by 60 ± 10%; this effect was dose dependent. The substitution of sodium with lithium, instead of choline, had no effect on the uptake process. $[^{125}I]T_4$ uptake was lower than that of $[^{125}I]T_3$ and not affected by sodium depletion. The half-maximal effect of sodium deprivation on $[^{125}I]T_3$ uptake was reached at an extracellular sodium concentration of about 40 mM. The variation of external pH influenced T_3 accumulation by thymocytes. $[^{125}I]T_3$ progressively decreased from acid to alkaline pH under normal and sodium-depleted conditions; however, the sodium-dependent fraction was more than doubled at physiological pH compared to that at more acidic and more alkaline pH. The sodium ionophore monensin decreased T_3 uptake by 51% at a concentration of 20 μM. These results indicated the existence of a sodium-related mechanism of T_3 uptake into mouse thymocytes that does not operate for T_4 uptake. (*Endocrinology* **129:** 2175–2179, 1991)

AN ACTIVE or facilitated transport of thyroid hormones (TH) into cells has been demonstrated in several tissues *in vitro* (1–11). In some of them it was shown that TH uptake also requires the presence of a sodium gradient (2, 4, 5, 11); this fact seems to indicate that THs enter the cells by a mechanism similar to that of transport system A for neutral amino acids, as suggested by Hennemann *et al.* (12). An involvement of sodium in TH entry was first demonstrated in hepatocytes by the sensitivity to ouabain of the initial rate of uptake (2). In our previous study in skeletal muscle we extended these findings by showing that T_3 uptake is dependent on extracellular sodium as well as sodium entry into cells, possibly related to Na^+/H^+ pump activity (11). However, there is no general consensus about the existence of a sodium-dependent mechanism of iodothyronine entry into cells. Recently, in fact, Blondeau *et al.* (13) and Topliss *et al.* (14) showed sodium-insensitive pathways for TH entry in cultured hepatocytes.

To test the hypothesis that the sodium traffic at the plasma membrane could be involved in these mechanisms, we investigated TH uptake into isolated mouse thymocytes; in these cells, 80% of the intracellular T_3 comes from plasma (15, 16), and they have a well characterized Na^+/H^+ pump at the plasma membrane level (17). Previous work from our laboratory indicated that T_3, but not T_4, enters the thymocytes by a saturable, specific, and energy-dependent mechanism, which was inhibited by ouabain (1). The aim of this study was, therefore, to investigate whether extracellular sodium and cationic movement across the plasma membrane may influence TH uptake into thymocytes.

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Address all correspondence and requests for reprints to: Marco Centanni, M.D., 2° Cattedra di Endocrinologia, c/o 2 Clinica Medica, Policlinico Umberto 1°, 00161 Rome, Italy.
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Materials and Methods

Cell preparation

BALB-c mice, 3–6 weeks old, were obtained from Charles River Ltd. (Como, Italy). Mice were killed by cervical dislocation, the thymus was removed, and thymocytes were prepared as described for rats by Segal and Ingbar (18) with some modification. Briefly, the thymus glands were washed in Krebs-Ringer (0.9% sodium chloride, 100 parts; 1.15% potassium chloride, 4 parts; 0.61% calcium chloride dihydrate, 3 parts; 2.11% potassium dihydrogen phosphate, 1 part; 3.82% magnesium sulfate eptahydrate, 1 part) and 25 mM Tris-HCl (KRT) buffer, pH 7.4, and finely minced. The cells were then filtered through a nylon mesh, centrifuged at 1200 rpm for 10 min at 4 C, and finally resuspended in the same buffer up to a concentration of 20 × 10^6 cells/ml. Cell viability was checked by trypan blue exclusion before and after the experiments; viability was greater than 95% in all conditions tested. Protein

content was measured by the method of Lowry *et al.* (19), using BSA as standard.

Uptake measurement

Isolated cells were equilibrated for 30 min at 37 C in KRT at physiological pH unless otherwise specified; incubation was carried out at 37 C in regular or sodium-depleted KRT buffer by adding 25 pM ^{125}I-labeled T_3 and T_4 to 1 ml cell suspension for the times and conditions specified in the figure legends. At the end of the incubation, 200-μl aliquots of each sample were layered over an equal volume of 30% sucrose in a Beckman microtube (Palo Alto, CA) and centrifuged at 13,000 rpm for 30 sec. The tips of the tubes were cut, and the radioactivity in the pellet was determined in a γ-counter. Negligible radioactivity was measured in the sucrose cushion when tested as a control. In low sodium experiments, Na^+ was replaced by equimolar amounts of choline or lithium, as indicated in the figure legends. Monensin was used only during preincubation and then removed to avoid interference with TH binding. The uptake of TH was measured as the percentage of total radioactivity per 10^6 cells. The nonspecific uptake was determined as the uptake of labeled TH in the presence of an excess (10 μM) of unlabeled T_3 and was used in each experiment as an internal control, then subtracted from the total uptake throughout the study. All experiments were carried out at least in triplicate. Statistical analysis was performed using Student's *t* test for unmpaired data, checking both upper and lower limits of the samples.

Chemicals

All chemicals used in this study were obtained from Sigma (St. Louis, MO). L-[^{125}I]T_3 (3300 μCi/μg) and L-[^{125}I]T_4 (4400 μCi/μg) were obtained from New England Nuclear (Florence, Italy). Freshly prepared, labeled THs were used throughout the study. The purity of labeled and unlabeled hormones was assayed by TLC on silica gel, using formic acid-methanol-chloroform (1:3:16), according to the method of Sato and Cahnmann (20). Negligible deiodination was present under all conditions tested.

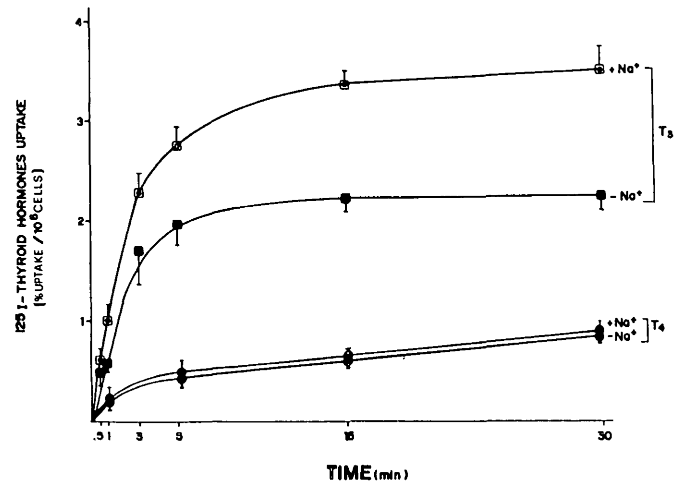

FIG. 1. Effect of sodium deprivation on the time course of labeled T_3 and T_4 uptake. Thymocytes (10–20 × 10^6 cells/ml) were equilibrated for 30 min at 37 C and incubated under the same conditions with [^{125}I] T_3 and [^{125}I]T_4 (25 pM) for the indicated times at pH 7.43 ± 0.05 in the presence of 140 mM Na^+ (⊡) or in its absence (■); when sodium was removed, choline was used to maintain osmolarity. The values are the mean ± SD of five different experiments.

TABLE 1. Effect of sodium deprivation on T_3 uptake at equilibrium

	n	% Uptake (×10^6 cells)	Change (%)	P^a	P^b
a) Control	5	3.56 ± 0.11[c]			
Na^+ depletion	5	2.24 ± 0.12	−37	<0.001	
b) Control + excess T_3[d]	3	1.53 ± 0.11	−57	<0.001	
Na^+ depletion + excess T_3	3	1.60 ± 0.08	−55	<0.001	NS

Cells were equilibrated and incubated for 30 min in KRT buffer (pH 7.45 ± 0.05) containing 20 pM [^{125}I]T_3 with or without 10 μM unlabeled T_3 in the presence or absence of 140 mM NaCl. n, Number of experiments in triplicate.

[a] *P* values calculated using a as reference.

[b] *P* values calculated using b as reference.

[c] Mean ± SD.

[d] 10 μM unlabeled T_3.

Results

The effect of Na^+ deprivation on [^{125}I]T_3 and [^{125}I]T_4 uptake in thymocytes is shown in Fig. 1. At equilibrium (1) 3.58 ± 0.26% of the added [^{125}I]T_3 was associated with 10^6 cells. The removal of extracellular sodium significantly reduced [^{125}I]T_3 uptake both during its linear phase (1 min) and at equilibrium; at 30 min, [^{125}I]T_3 uptake was reduced by 37% compared to the control uptake ($P < 0.001$). At the same time the unsaturable uptake, that is uptake in the presence of a large excess of unlabeled T_3 (1), represented 43 ± 5% of the total uptake. The effects of sodium depletion and excess unlabeled T_3 were not additive, indicating that sodium affects only the saturable uptake (Table 1). The uptake of [^{125}I]T_4 was lower than that of T_3 and was not affected by sodium depletion (Fig. 1). In Table 2 are shown the effects of sodium substitution with two different cations and the effect of monensin on specific [^{125}I]T_3 uptake at equilibrium. When sodium was replaced by choline, specific [^{125}I]T_3 uptake was reduced by 60%; however, after preincubation in choline medium, the incubation with sodium chloride restored uptake to normal, indicating that choline chloride had no intrinsic effect on T_3 uptake. When Na^+ was replaced by lithium, which, unlike choline, is a substrate for the Na^+/H^+ pump, [^{125}I]T_3 uptake was not different from the control (Table 2), showing that lithium can substitute sodium in the process. To investigate the possible involvement of the Na^+/H^+ antiporter system, we studied the effect of monensin, an ionophore that activates sodium-dependent H^+ release by increasing sodium entry into thymocytes (17). The

TABLE 2. Effects of cations and monensin on specific T_3 uptake

Salt used[a]		n	% Uptake ($\times 10^6$ cells)	Change (%)	*P*
Preincubation	Incubation				
NaCl	NaCl	6	1.99 ± 0.12[b]		
ChCl	ChCl	4	0.80 ± 0.10	−60	<0.001
ChCl	NaCl	3	1.81 ± 0.14	−9	NS
LiCl	LiCl	3	1.75 ± 0.17	−12	NS
NaCl + monensin (1 μM)	NaCl	3	2.01 ± 0.11		NS
NaCl + monensin (10 μM)	NaCl	3	1.38 ± 0.05	−31	<0.05
NaCl + monensin (20 μM)	NaCl	6	0.98 ± 0.08	−51	<0.001

Cells were equilibrated for 30 min at 37 C in the presence or absence of sodium, replaced by either isoosmotic choline or lithium. Cells were then centrifuged, resuspended, and incubated for 30 min at 37 C in the presence of 25 pM labeled T_3. n is the number of experiments.

[a] Salt (140 mM) present in KRT and its modification.

[b] Mean ± SD.

results indicated that the effect was dose related, and the maximal concentration of monensin used (20 μM) decreased $[^{125}I]T_3$ uptake by 51% (Table 2).

The specific $[^{125}I]T_3$ uptake has been proven to correlate with the extracellular sodium concentration. During incubation, the cells were exposed to different concentrations of sodium (Fig. 2). The reduction of sodium in the medium was paralleled by a decreased $[^{125}I]T_3$ uptake; the half-maximal effect was reached at an extracellular Na^+ concentration of about 40 mM. We should point out that sodium depletion was not necessary to obtain the maximal reduction of T_3 uptake. This finding suggests that a crucial Na^+ concentration is required to activate the process.

A previous report indicated that in thymocytes, extracellular pH interferes with sodium movements through the proton pump (17); therefore, we investigated the effects of different extracellular pH values on $[^{125}I]T_3$ uptake under normal and sodium-depleted conditions. The total $[^{125}I]T_3$ uptake was sensitive to extracellular pH, progressively decreasing from pH 6.5 to pH 8.5, whereas the absolute amount of unsaturable uptake was similar at each pH studied; thus, the effect of pH was exerted on the saturable uptake (not shown). The specific saturable $[^{125}I]T_3$ uptake was similar at pH 6.5 and 7.5 and then decreased to 38% of the control value at pH 8.5 (Fig. 3). The sodium depletion reduced $[^{125}I]T_3$ uptake at every pH studied, but in dissimilar amounts; the reduction was noticeable at pH 7.5 and almost disappeared at pH 8.5 (Fig. 3). As a result, after subtraction of the

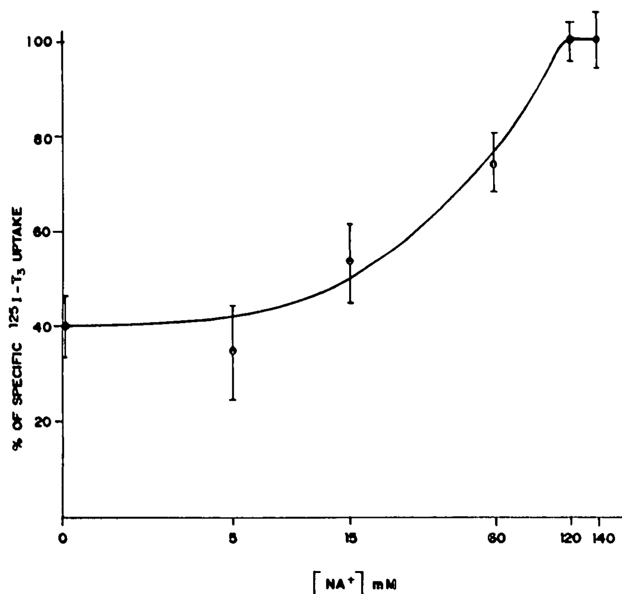

FIG. 2. Specific T_3 uptake as a function of extracellular sodium. Cells were equilibrated for 30 min in a choline buffer at 37 C and then incubated for 30 min with $[^{125}I]T_3$ (25 pM), pH 7.46 ± 0.05 in the presence of increasing concentrations of extracellular sodium, using choline to maintain the isoosmolarity of the samples. The results are the mean ± SD of four separate experiments.

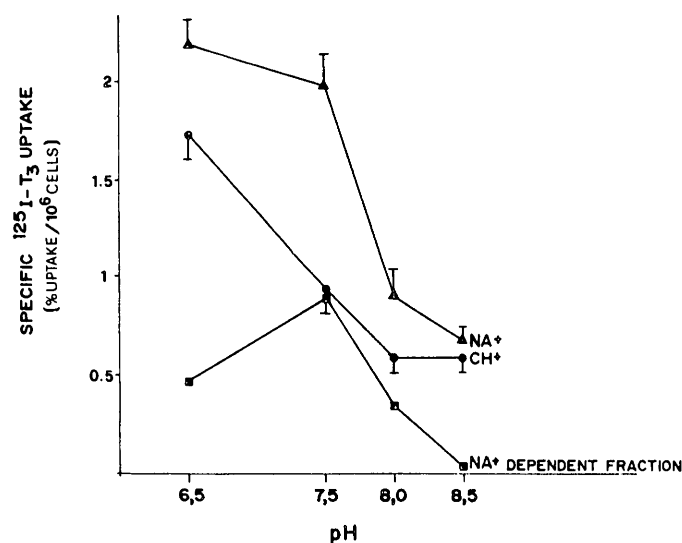

FIG. 3. Effect of pH on specific T_3 uptake in the presence or absence of extracellular sodium. Thymocytes (10–20 × 10^6 cells/ml) were equilibrated for 30 min at 37 C and incubated with labeled T_3 (25 pM) at the indicated pH in the presence (▲) or absence (●) of extracellular sodium replaced by isoosmotic choline (CH^+). The sodium-dependent fraction (■) was calculated by subtracting the values obtained in the absence of sodium from that obtained in its presence. The results are the mean ± SD of five different experiments.

sodium-independent portion, the sodium-sensitive fraction was more than doubled ($P < 0.001$) at physiological pH compared to that at more acidic and/or more alkaline pH (Fig. 3); this suggests the existence of a sodium-sensitive mechanism that is mainly active at physiological pH. The uptake of $[^{125}I]T_4$ at different pH values was similar, but was lower than T_3 uptake and not affected by sodium depletion at any pH studied (data not shown).

Discussion

The results in the present study are consistent with the existence of sodium-sensitive $[^{125}I]T_3$ uptake into mouse thymocytes. The fact that Na^+ traffic is related to the activity of the cell membrane pumps (Na^+/H^+ and Na^+/K^+) indicates that this ion may represent the linkage between the T_3 entry mechanism and energy-related processes, as suggested by Christensen (21) for some amino acid transport systems. As in skeletal muscle (11), Na^+ deprivation reduced $[^{125}I]T_3$ uptake in mouse thymocytes; the effect was dose related, and the process was sensitive to monensin. As in muscle, $[^{125}I]T_4$ uptake was not affected by sodium depletion.

Some researchers have demonstrated a sodium-insensitive pathway for TH entry in cultured hepatocytes (12–14) and tadpole red blood cells (3). Moreover, it has been suggested that the transport of THs in erythrocytes is related to the amino acid transport system T (22), while the transport of T_4 in neuroblastoma cells is related to system L (23); both systems are Na^+ independent. Our present results indicate that the system that mediates $[^{125}I]T_3$ uptake in thymocytes is not inhibited by lithium and is only partly sensitive to extracellular sodium availability. This behavior is typical of the amino acid transport systems; different independent mechanisms may transport the same amino acid depending on the different environmental conditions and the different tissues studied. For example, neutral amino acids transported by the Na^+-dependent system A (24) could be taken up by the Na^+-independent system L by changing the external pH (25). On the same line, the ability to accept lithium as a substitute for sodium is a typical feature of the Na^+-dependent amino acid transport system A, but not of the very similar ASC (25); this suggests that the ability of lithium to substitute sodium is restricted to certain mechanisms and tissues. In thymocytes, lithium is a substrate for the proton pump, although it is less efficient than sodium (17). This may explain the lack of $[^{125}I]T_3$ uptake inhibition when lithium substitutes sodium in the medium. These findings may also explain the difference from our previous results in muscle, where the sodium-dependent uptake was equal to the specific uptake and was inhibited by lithium (11). In the present study monensin inhibited $[^{125}I]T_3$ uptake; in thymocytes this ionophore increases sodium entry through the proton pump (17) and the Na^+ channels (26, 27). Thus, similarly to ouabain (1), its inhibitory effect could be explained by the disappearance of the sodium gradient mediated by the activity of membrane pumps. However, due to the complexity and tissue specificity of the Na^+/H^+ pump activity (17, 26, 27), the interpretation of the monensin effect has to be restricted to the tissue studied, since, as in the case of lithium, different results were obtained in other tissues (11, 14).

In the present report external pH has been proven to affect $[^{125}I]T_3$ uptake by thymocytes both under Na^+-depleted and undepleted conditions. A similar effect was reported by others in different tissues (8, 13), where external pH affected the Na^+-insensitive transport systems. In the present study sodium deprivation decreased $[^{125}I]T_3$ uptake at every extracellular pH studied. Moreover, the Na^+-dependent fraction was maximal at pH 7.5; thus, at this pH, the reduction of specific T_3 uptake represents mainly the reduction of the sodium-sensitive fraction. This suggests the existence of a specific sodium-sensitive mechanism that is active at physiological pH, confirming that more than one mechanism of T_3 uptake may be involved.

In conclusion, this study indicates that $[^{125}I]T_3$ uptake into thymocytes is in part sodium dependent and involves a mechanism related to the distribution of cations across the membrane. Since $[^{125}I]T_4$ uptake in this tissue is not sodium sensitive, a further difference in the TH entry process has been demonstrated.

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