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Uptake of Triiodothyroacetic Acid and Its Effect on Thyrotropin Secretion in Cultured Anterior Pituitary Cells*

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ABSTRACT

The uptake of [^{125}I]triiodothyroacetic acid ([^{125}I]Triac) in anterior pituitary cells was investigated and compared with that of [^{125}I]T_3. Furthermore, the effects of Triac, T_3, and T_4 on TSH release were compared. Cells isolated from adult male Wistar rats were cultured for 3 days in medium with 10% fetal calf serum. Uptake was measured at 37 C with [^{125}I]Triac (100,000 cpm; 120 pM) or [^{125}I]T_3 (50,000 cpm; 50 pM) in medium with 0.5% BSA. In this medium, the ratio of the free fractions of Triac, T_3, and T_4 was 1:8:1. Exposure of cells to 100 nM TRH for 2 h stimulated TSH release by 80–110% ($P < 0.001$). Comparing total hormone levels (1 nM to 1 μM), Triac and T_3 were equally effective in reducing this response, and both were 10-fold more effective than T_4. The time course (15 min to 4 h) of [^{125}I]Triac uptake was similar to that of [^{125}I]T_3, showing equilibrium after 1 h. Unlabeled Triac (1 μM) reduced the uptake of [^{125}I]Triac and [^{125}I]T_3 at all time intervals. Expressed per pM free hormone, the cellular and nuclear uptake of [^{125}I]Triac were twice those of [^{125}I]T_3. The 15-min uptake of [^{125}I]Triac was reduced by incubation with 10 nM unlabeled Triac (35%; $P < 0.001$). Maximum inhibition (56%; $P < 0.001$) was found with 10 μM Triac. A similar effect was seen with 10 μM T_3, T_4, or 3,3′,5,5′-tetraiodothyroacetic acid. Preincubation (30 min) and incubation (15 min) with 10 μM oligomycin reduced the cellular ATP content by 51% ($P < 0.001$), [^{125}I]T_3 uptake by 77% ($P < 0.001$), and [^{125}I]Triac uptake by only 25% ($P < 0.001$). The temperature dependence of [^{125}I]Triac and [^{125}I]T_3 uptake was the same. Preincubation and incubation with 10 μM monensin (reduces the Na^+ gradient) or 10 μM monodansylcadaverine (inhibits receptor-mediated endocytosis) reduced 15-min [^{125}I]Triac uptake by 15% ($P < 0.005$) and 19% ($P < 0.005$), respectively. The data show that 1) Triac, on the basis of the free hormone concentration, is more potent than T_3 or T_4 in suppressing TSH secretion; and 2) the rapid uptake of [^{125}I]Triac by the anterior pituitary occurs by a carrier-mediated mechanism that is only partially dependent on ATP or the Na^+ gradient. (*Endocrinology* **135:** 2700–2707, 1994)

THE METABOLISM of T_3 occurs by three pathways: deiodination, conjugation, and side-chain alteration. Whereas 50–60% of T_3 in humans is deiodinated to 3,3′-diiodothyronine (1), alteration of the alanine side-chain, resulting in the production of 3,3′,5-triiodothyroacetic acid (Triac), may account for around 14% of T_3 metabolism in man (2). However, under pathological conditions, as, for example, fasting, Triac production is substantially increased (3, 4). Furthermore, these researchers suggested that Triac might be of importance for the suppression of TSH secretion in fasting, which occurs despite low plasma T_3 and T_4 levels. Also, under conditions other than fasting, characterized by low plasma TSH, T_3, and T_4, Triac might play a role in the suppression of TSH secretion (5).

In euthyroid and hypothyroid subjects, administration of Triac suppresses basal TSH secretion (6–8) as well as the TSH response to TRH (6, 9). Furthermore, Triac is used in the treatment of patients with thyroid cancer (10, 11) and generalized resistance to thyroid hormones (12, 13). However, the question of whether the effects of Triac are specific for the pituitary gland has not been definitively answered (8, 9, 11, 14).

Early *in vitro* studies showed that T_3 and Triac were equally potent in the suppression of TSH production and displacement of [^{125}I]T_3 from the nuclear binding sites in mouse thyrotropic tumor cells (15). In rat pituitary fragments superfused with BSA-containing medium, both 0.1 μM Triac and 0.1 μM T_3 reduced the TRH-induced TSH release by around 25% (16).

Previously, we reported evidence for carrier-mediated uptake of T_3 and T_4 in cultured anterior pituitary cells of euthyroid rats (17, 18). Moreover, T_3 and T_4 seem to share the same carrier in the pituitary (18, 19). The purpose of the present work was to study 1) the effect of Triac, compared to those of T_3 and T_4, on basal and TRH-induced TSH release; and 2) the mechanism of uptake of [^{125}I]Triac into cultured anterior pituitary cells. Furthermore, we questioned whether the uptake of [^{125}I]Triac occurred by the same transport mechanism as that described for T_3 and T_4.

Materials and Methods

Materials

All solutions used for cell isolation and cell culture were obtained from Gibco Europe (Breda, The Netherlands), with the exception of

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human serum albumin (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), dispase (grade II; Boehringer, Mannheim, Germany), absorbed fetal calf serum (Flow Laboratories, Irvine, Scotland), and Fungizone (Bristol-Myers Squibb, Woerden, The Netherlands). Culture dishes (48 wells) were obtained from Costar (Cambridge, MA). All iodothyronines, 3,5-diiodothyroacetic acid Triac, and 3,3',5,5'-tetraiodothyroacetic acid (Tetrac), were obtained from Henning Berlin (Berlin, Germany). Piperazine-*N,N'*-bis-[2-ethane sulfonic acid], HEPES, *N,N*-bis-[2-hydroxyethyl]2-aminoethane sulfonic acid, BSA (fraction V), monensin, oligomycin, and monodansylcadaverine (MDC) were purchased from Sigma Chemical Co. (St. Louis, MO). TRH was obtained from Hoechst (Frankfurt am Main, Germany). [3'-^{125}I]T_3 (3070 μCi/μg) and carrier-free Na^{125}I were purchased from Amersham International (Aylesbury, United Kingdom). [3'-^{125}I]Triac (2730 μCi/μg) was prepared from Na^{125}I and 3,5-diiodothyroacetic acid using the chloramine-T method (20). Sephadex LH-20 and G-25 were obtained from Pharmacia (Uppsala, Sweden). All other reagents were of the highest purity available.

Animals

All experiments were performed using male Wistar rats, weighing 220–250 g. The animals had free access to food and water and were kept in a controlled environment (21 C) with constant day length (12 h).

Cell culture

Animals (12–18 for each experiment) were killed between 0900–0930 h by decapitation. The pituitary glands were removed within 5 min, the neurointermediate lobe was discarded, and the anterior lobes were collected in calcium- and magnesium-free Hanks' Balanced Salt Solution supplemented with 10 g/liter human serum albumin, penicillin (10^5 U/liter), Fungizone (0.5 mg/liter), and sodium bicarbonate (0.4 g/liter). Anterior pituitary cells were dissociated with dispase (final concentration, 2.4×10^3 U/liter), as described in detail previously (21). From each pituitary, around 1.5×10^6 cells were obtained, and the viability of the cells, determined by trypan blue exclusion, was greater than 90%.

The cells were cultured at 37 C in a water-jacketed incubator with 5% CO_2 at a density of 5–8 $\times$ 10^5 cells/well in 48-well culture dishes. The cells had attached to the wells after 2 days of culture. On day 3, the cells were used for experiments. The culture medium consisted of Minimum Essential Medium with Earle's salts supplemented with nonessential amino acids, sodium pyruvate (1 mmol/liter), 10% fetal calf serum (in experiments for Table 1, 10% absorbed fetal calf serum), penicillin (10^5 U/liter), Fungizone (0.5 mg/liter), L-glutamine (2 mmol/liter), and sodium bicarbonate (2.2 g/liter), pH 7.4 (21, 22).

Cellular uptake of [^{125}I]Triac and [^{125}I]T_3

After removal of the culture medium, cells were preincubated with 0.5 ml incubation medium. The incubation medium was identical to the culture medium, except that the fetal calf serum was replaced by 0.1% or 0.5% BSA. Preincubation was carried out for 30 min at 37 C in the absence or presence of 10 μM oligomycine, MDC, or monensin. The medium was removed after preincubation, and incubation was started with 0.25 ml medium containing the same additions as those described above, 10 nM to 10 μM Triac, or 10 μM T_3, T_4, or Tetrac and in all cases [^{125}I]Triac (100,000–200,000 cpm; 120–240 pM) or [^{125}I]T_3 (50,000 cpm; 50 pM).

Incubations lasting for more than 1 h were performed at 37 C in humidified air with 5% CO_2. Incubations of shorter duration took place in a 37 C incubation chamber on a rotating device without CO_2. Therefore, the $NaHCO_3$ in the culture medium was replaced by an equimolar amount of HEPES (8.9 mM), piperazine-*N,N'*-bis-[2-ethane sulfonic acid] (10.6 mM) and *N,N*-bis-[2-hydroxyethyl]2-aminoethane sulfonic acid (11.2 mM).

After incubation, the medium was removed, and the cells were washed with 1-ml volumes of ice-cold saline to remove tracer not bound to the cells. Cells were dissolved in 1 ml 0.1 N NaOH and counted for ^{125}I activity in a 16-channel γ-counter (NE 1600, Nuclear Enterprises, Edinburgh, Scotland). The amount of [^{125}I]Triac or [^{125}I]T_3 taken up was expressed as a percentage of the added radioactivity (percentage of the dose). The same procedure was applied to incubations without cells (blanks). All results were corrected for the amount of radioactivity retained in the wells without cells.

Nuclear binding of [^{125}I]Triac and [^{125}I]T_3

After incubation, cells were washed once with ice-cold saline (0.9% NaCl). The cells were harvested from the wells with a rubber policeman in 1 ml PBS (on ice) and counted for 30 sec. Isotope uptake, calculated as a percentage of the dose with this method, gave similar values for the cellular radioactivity as the usual procedure with 0.1 N NaOH. All of the following procedures were performed on ice. A cell pellet was obtained after centrifugation (300 $\times$ *g*; 4 C; 7 min), counted (30 sec), and solubilized in 1 ml PBS containing 0.5% Triton X-100, as previously described (23). After 2 min of continuous vortexing, nuclei were spun down (900 $\times$ *g*; 4 C; 5 min) and washed once with 1 ml PBS containing 0.5% Triton X-100. The nuclear pellets were counted for 5 min.

LH-20 chromatography

Aliquots of the incubation medium were chromatographed on Sephadex LH-20 (24). Iodide was eluted from the column with 4 $\times$ 1 ml 0.1 N HCl. Subsequently, possible conjugates were eluted eight times with 1 ml H_2O, and finally, the remaining Triac or T_3 was eluted from the column four times with 1 ml 50% ethanol in 0.1 N NaOH.

TSH release and cellular content

After removal of the culture medium, cells were washed once with incubation medium. The culture medium was centrifuged (2000 $\times$ *g*), and the supernatant was frozen until hormone determination. The incubation medium was identical to the culture medium, except that fetal calf serum was replaced by 0.5% BSA. The pituitary cells were preincubated for 2 h at 37 C in the absence or presence of variable concentrations (1 nM to 1 μM) Triac, T_3, or T_4. The preincubation medium was discarded, and fresh incubation medium was added which contained 100 nM TRH without or with the same concentrations Triac, T_3, or T_4 as those described above. Incubation was continued for 2 h at 37 C. This medium was removed, centrifuged (2000 $\times$ *g*), and stored at −20 C.

To determine the TSH content of cells, 0.5 ml incubation medium was added to the wells. The cells were scraped from the wells with a rubber policeman, and the wells were sonicated twice for 30 sec. The extracts were removed, and the wells were washed once with 0.5 ml medium. The two fractions were then combined (volume of cell extract, 1.0 ml) and centrifuged (2000 $\times$ *g*) at room temperature, and the supernatant was frozen until further analysis.

TSH determination

TSH was measured by RIA. TSH (2 μg) was labeled with ^{125}I (1 mCi) by the lactoperoxidase method and subsequently purified on a Sephadex G-25 (medium) and a Sephadex G-100 column, as previously described (17).

Free Triac and T_3 concentrations

Calculation of the free Triac and free T_3 concentrations was based on determination of the free fractions by equilibrium dialysis (25). Determination of the free Triac fraction was corrected for the fact that only between 55–65% of [^{125}I]Triac is precipitated with $MgCl_2$.

In medium with 0.5% BSA, the free fractions of Triac, T_3, and T_4 were 0.47 $\pm$ 0.03% (n = 5), 3.45 $\pm$ 0.05% (n = 4), and 0.41 $\pm$ 0.03% (n = 5), respectively. In rat serum, the free fractions of Triac, T_3, and T_4 were 0.028%, 0.381%, and 0.025%, respectively. The free Triac or T_3 fraction in 0.5% BSA did not change after the addition of 10 nM or 1 μM unlabeled Triac.

In medium with 0.1% BSA, the free fraction of Triac was 1.84 $\pm$ 0.07% (n = 9). This value did not change after the addition of 10 nM

Triac (1.75 ± 0.06%; n = 5), but it increased after the addition of 10 μM Triac, T_3, T_4, or Tetrac to 2.55 ± 0.07% (n = 5), 2.04 ± 0.05% (n = 3), 2.15 ± 0.06% (n = 3), and 2.42% (n = 2), respectively. The free Triac fractions (in 0.1% BSA) in the presence of 10 μM MDC, oligomycin, or monensin were 1.77 ± 0.07% (n = 5), 1.76 ± 0.10% (n = 5), and 1.83 ± 0.13% (n = 5), respectively.

ATP determination

The cellular ATP content was determined in perchloric acid (0.2 M) extracts with the Lumac A.E.C.-Kit (Lumac, Landgraaf, The Netherlands).

Statistics

The statistical significance of the effects of the tested compounds on [^{125}I]Triac and [^{125}I]T_3 uptake or on TSH secretion was evaluated by one-way analysis of variance and Student's *t* test for unpaired observations. $P < 0.05$ was regarded as statistically significant.

Results

TSH release during culture

To determine whether T_3, T_4, or Triac affected basal TSH release from cultured anterior pituitary cells, these three hormones were added at a concentration of 10 nM to cells cultured in medium supplemented with absorbed fetal calf serum. In addition, the effects of rT_3 (10 nM) and TRH (1 nM) were tested (Table 1). The inhibitory effect of the thyroid hormones or analogs on basal TSH secretion during 3 days of culture decreased in the order Triac > T_3 > T_4 > rT_3. The presence of 1 nM TRH almost doubled TSH release. In two separate experiments, it was found that total TSH (released into the medium plus cellular content) increased by 65% ($P < 0.001$) after culture in the presence of 1 nM TRH (not shown).

TSH release during short term exposure to TRH

The effects of Triac, T_3, and T_4 were also compared with respect to inhibition of TRH-induced TSH release during short (2-h) incubation experiments. As shown in Table 2, TSH release increased by around 80% after exposure to 100 nM TRH. At the same time, the cellular TSH content decreased proportionally, suggesting that TRH stimulated the release of stored TSH (not shown). The stimulating effect of TRH on TSH release was almost completely blocked by preincubation and incubation with 10 nM or 1 μM T_3 or Triac (Table 2).

In two experiments, we compared the effects of Triac and T_4 at a concentration of 25 nM on TRH-induced TSH release. TSH release (4.20 ± 0.37 ng; n = 9) was stimulated 2-fold after exposure to 100 nM TRH (8.73 ± 0.25 ng; n = 9; +108%; $P < 0.001$). After preincubation for 2 h with 25 nM Triac or T_4, TSH release was 4.92 ± 0.25 ng (n = 6) and 5.96 ± 0.49 ng (n = 6), respectively ($0.05 < P < 0.10$).

Finally, we tested within one experiment the effects of Triac, T_3, and T_4 on TRH-induced TSH release at lower concentrations than those of the previous series. The results shown in Table 3 demonstrate that at concentrations of 1, 2.5, and 10 nM, T_3 and Triac were equally potent in suppression of the TRH-induced TSH release. T_4, however, had no significant inhibitory effect at a concentration of 2.5 nM. The effect of T_4 increased with higher concentrations, and the effect of 100 nM T_4 was approximately as large as that of 10 nM T_3 or Triac.

Time course of [^{125}I]Triac and [^{125}I]T_3 uptake

Figure 1 shows the time course of uptake of [^{125}I]Triac (Fig. 1A) compared with that of [^{125}I]T_3 (Fig. 1B) in cultured anterior pituitary cells. The uptake of the two isotopes was also measured in the presence of 1 μM unlabeled Triac (*open symbols* in Fig. 1, A and B). The uptake of [^{125}I]Triac and [^{125}I]T_3 showed a steep phase up to 1 h of incubation. Between 1 and 4 h of incubation, the uptake of [^{125}I]T_3 showed some further increase, whereas that of [^{125}I]Triac had reached the maximum level.

The presence of 1 μM unlabeled Triac significantly reduced the uptake of both [^{125}I]Triac and [^{125}I]T_3 at any time point,

TABLE 1. Effects of Triac, iodothyronines, and TRH on TSH release during culture of anterior pituitary cells

Exp Conditions	TSH release (ng): addition present during	
	3 days of culture	Last day of culture
Controls	37.2 ± 0.8 (9)	30.6 ± 0.4 (8)
10 nM T_3	31.1 ± 0.5 (6)[a]	26.6 ± 0.1 (3)[b]
10 nM T_4	34.0 ± 0.5 (3)	28.8 ± 0.8 (3)
10 nM Triac	28.7 ± 1.2 (3)[a]	27.4 ± 1.6 (3)[c]
10 nM rT_3	35.6 ± 1.7 (3)	32.6 ± 1.0 (3)
1 nM TRH	71.7 ± 3.7 (3)[a]	ND

Data represent the mean ± SE of three to nine observations in two independent experiments. Anterior pituitary cells (500,000 cells/well) were cultured for 3 days in culture medium containing 10% absorbed fetal calf serum. Hormones were added either at the start of culture (*first column*) or after 2 days of culture (*last column*) at the indicated concentrations. TSH in the culture medium was measured by RIA. ND, Not determined.

[a] $P < 0.001$ *vs.* controls.

[b] $P < 0.005$.

[c] $P < 0.01$.

TABLE 2. Short term effects of T_3 and Triac on TRH-induced TSH release from cultured anterior pituitary cells

Exp Conditions	TSH Release (ng)	Stimulation (%)	*P*
Controls (n = 12)	3.37 ± 0.07		
+ TRH (100 nM) (n = 12)	6.07 ± 0.05	+80	<0.001[a]
TRH + 10 nM T_3 (n = 5)	3.87 ± 0.15	+15	<0.001[b]
TRH + 1 μM T_3 (n = 6)	3.05 ± 0.09	−9	<0.001[b]
TRH + 10 nM Triac (n = 9)	3.42 ± 0.06	+1	<0.001[b]
TRH + 100 nM Triac (n = 9)	3.43 ± 0.10	+2	<0.001[b]
TRH + 1 μM Triac (n = 9)	3.01 ± 0.04	−11	<0.001[b]

Data indicate the mean ± SE of 5–12 observations from 3 independent experiments. Anterior pituitary cells were cultured for 3 days at a density of 500,000 cells/well. The cells were preincubated for 2 h in the absence or presence of T_3 or Triac at concentrations of 10 nM to 1 μM. Then, they were incubated with TRH (100 nM) for 2 h without or with the additions indicated. TSH was measured in the incubation medium by RIA.

[a] *Vs.* controls.

[b] *Vs.* TRH alone.

TABLE 3. Comparison of the short term effects of Triac, T_3, and T_4 on the TRH-induced TSH release from cultured anterior pituitary cells

Exp Conditions	TSH release (ng)	Stimulation (%)	*P*
Controls (6)	3.97 ± 0.35		
+ 100 nM TRH (6)	7.24 ± 0.22	+82	<0.001[a]
TRH + 1 nM T_3 (3)	5.64 ± 0.25	+42	<0.001[b]
TRH + 2.5 nM T_3 (3)	5.65 ± 0.43	+42	<0.001[b]
TRH + 10 nM T_3 (3)	5.50 ± 0.13	+39	<0.001[b]
TRH + 1 nM Triac (3)	5.73 ± 0.15	+44	<0.001[b]
TRH + 2.5 nM Triac (3)	5.79 ± 0.26	+46	<0.001[b]
TRH + 10 nM Triac (3)	5.47 ± 0.15	+38	<0.001[b]
TRH + 100 nM Triac (3)	4.96 ± 0.12	+25	<0.001[b,c]
TRH + 2.5 nM T_4 (3)	6.70 ± 0.30	+69	NS[b]
TRH + 10 nM T_4 (3)	6.44 ± 0.09	+62	<0.05[b,d]
TRH + 100 nM T_4 (3)	5.32 ± 0.32	+34	<0.001[b]
TRH + 1 μM T_4 (3)	4.37 ± 0.47	+10	<0.001[b]

Data show the mean ± SE of three to six observations in a single experiment. Anterior pituitary cells were cultured for 3 days at a density of 500,000 cells/well. The experiment was performed exactly as described in Table 2.

[a] *Vs.* controls.

[b] *Vs.* TRH alone.

[c] $P < 0.05$ or less *vs.* TRH plus 1 or 2.5 nM Triac.

[d] $P < 0.05$ or less *vs.* TRH plus 10 nM Triac or 10 nM T_3.

and the effect was largest after 4 h [89% for [^{125}I]Triac ($P < 0.001$; n = 6) and 63% for [^{125}I]T_3 ($P < 0.001$; n = 6)]. It is evident from the *upper curves* in Fig. 1, A and B, that the uptake, expressed as a percentage of the dose of [^{125}I]T_3, was 3-fold higher than that of [^{125}I]Triac. One important factor that explains this difference is the different free fraction of the two isotopes in buffer with 0.5% BSA, *i.e.* 0.47% for [^{125}I]Triac and 3.45% for [^{125}I]T_3. By expressing the uptake data per pM free hormone, the curves presented in Fig. 1C were obtained, which clearly shows that the uptake of [^{125}I]Triac was actually more than twice that of [^{125}I]T_3.

The media from the 4-h incubations were chromatographed on LH-20 columns to determine whether [^{125}I]T_3 or [^{125}I]Triac was metabolized by the pituitary cells. In the media of incubations with [^{125}I]T_3 (n = 3), 99.9 ± 3.5% of the radioactivity was recovered in the iodothyronine fraction. In the media of incubations with [^{125}I]Triac (n = 3), 100.5 ± 2.1% (n = 3) was recovered in the iodothyronine fraction (not shown).

The effect of 10 nM unlabeled Triac on the uptake of [^{125}I]Triac and [^{125}I]T_3 was tested in incubations lasting 1 h. Again, it can be seen in Fig. 2 that the uptake of [^{125}I]Triac (n = 6) was 3-fold greater than that of [^{125}I]T_3 (n = 6; $P < 0.001$). The presence of 10 nM unlabeled Triac, which did not change the free fraction of [^{125}I]Triac or that of [^{125}I]T_3, reduced the uptake of [^{125}I]Triac by 67% ($P < 0.001$), but reduced that of [^{125}I]T_3 by only 35% ($P < 0.001$).

Nuclear binding of [^{125}I]Triac and [^{125}I]T_3

To assess nuclear binding of T_3 and Triac, anterior pituitary cells (800,000 cells/well) were incubated for 1 h with [^{125}I]Triac or [^{125}I]T_3 in the absence or presence of 10 μM unlabeled

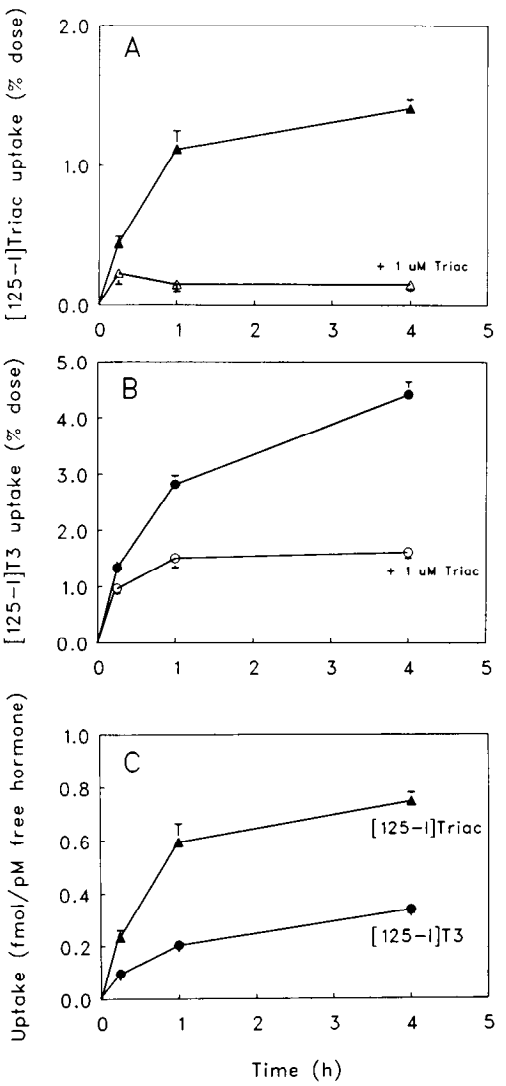

FIG. 1. Time course of uptake of [^{125}I]Triac (A) and [^{125}I]T_3 (B) in cultured anterior pituitary cells in the absence (▲ and ●) or presence (△ and ○) of 1 μM unlabeled Triac. In C, the uptake of [^{125}I]Triac and [^{125}I]T_3 is expressed per pM free hormone. Cells were cultured for 3 days at a density of 500,000 cells/well. After removal of the culture medium, they were preincubated for 30 min in incubation medium with 0.5% BSA. Thereafter, they were incubated in the same medium for periods of 15 min, 1 h, or 4 h with [^{125}I]Triac (100,000 cpm) or [^{125}I]T_3 (50,000 cpm) without or with unlabeled Triac, as described in *Materials and Methods*. Data represent the mean ± SE of six observations from two independent experiments.

Triac or T_3. To increase the free Triac fraction and, thus, the availability of isotope to the cells, [^{125}I]Triac uptake was measured in buffer with 0.1% BSA in this experiment. As shown in the *first column* of Table 4, the total uptake (percentage of the dose) of [^{125}I]Triac and [^{125}I]T_3 under these different incubation conditions was approximately the same. Again, the presence of unlabeled Triac showed a greater inhibitory effect on the cellular uptake of [^{125}I]Triac than on that of [^{125}I]T_3. Also, the inhibitory effect of unlabeled T_3 was greater on cellular [^{125}I]Triac uptake than on [^{125}I]T_3 uptake (Table 4, *second column*). The nuclear pellet (Table 4, *third column*) contained roughly the same amounts of [^{125}I]Triac

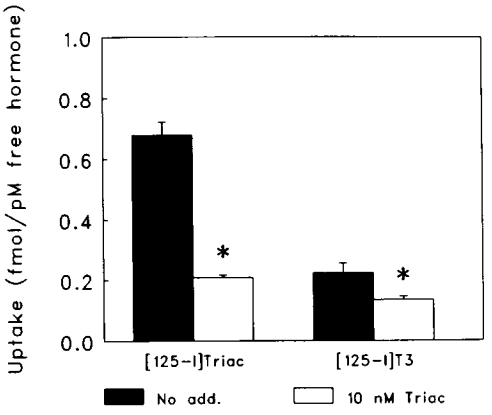

FIG. 2. Uptake of [^{125}I]Triac and [^{125}I]T_3 by cultured anterior pituitary cells in the absence or presence of 10 nM unlabeled Triac. Cells were cultured for 3 days at a density of 500,000 cells/well. Preincubation (30 min) and incubation (60 min) were performed as described in Fig. 1, except that half of the incubations contained 10 nM unlabeled Triac. *Bars* represent the mean ± SE of six observations from two independent experiments. *, $P < 0.001$, 10 nM Triac *vs.* no additions.

TABLE 4. Total and nuclear binding of [^{125}I]Triac and [^{125}I]T_3 in cultured anterior pituitary cells

Exp Conditions	[^{125}I]Radioactivity (% dose)		
	Total uptake	Cell pellet	Nuclear pellet
[^{125}I]Triac	4.87	2.67	0.84
+10 μM Triac	1.41	0.52	0.02
+10 μM T_3	1.32	0.50	0.02
[^{125}I]T_3	4.40	2.31	0.70
+10 μM Triac	3.07	1.17	0.03
+10 μM T_3	2.39	0.96	0.05

Data show the means of triplicate observations in a single experiment. Anterior pituitary cells were cultured for 3 days at a density of 800,000 cells/well. Preincubation (30 min) and incubation (1 h) for determination of [^{125}I]Triac uptake (200,000 cpm) were carried out in medium with 0.1% BSA, and for determination of [^{125}I]T_3 uptake (100,000 cpm) in medium with 0.5% BSA. Unlabeled Triac or T_3 (10 μM) was only present during incubation. Total uptake was measured in cells scraped from the wells in 1 ml PBS. After centrifugation of the suspension, a cell pellet was obtained and counted. The nuclear pellet was obtained after treatment with Triton X-100 (for further details, see *Materials and Methods*).

and [^{125}I]T_3 after 1 h (31% and 30% of the radioactivity in the cell pellets, respectively), and the nuclear binding of [^{125}I]Triac or [^{125}I]T_3 was almost completely blocked by simultaneous incubation with unlabeled Triac or T_3. Relative to the free fraction in the incubation medium, the nuclear uptake of [^{125}I]Triac was about twice that of [^{125}I]T_3 (0.84% dose with a free fraction of 1.84% for [^{125}I]Triac and 0.70% dose with a free fraction of 3.45% for [^{125}I]T_3).

Plasma membrane uptake of [^{125}I]Triac

To evaluate the uptake of [^{125}I]Triac at the level of the pituitary plasma membrane, experiments were performed in a manner similar to that previously described for [^{125}I]T_3 and [^{125}I]T_4 with 15 min of incubation (17, 18). With 0.5% BSA in the medium, the 15-min uptake of [^{125}I]Triac amounted to 0.46 ± 0.04% dose (n = 8), and with 0.1% BSA in the medium, it was 1.70 ± 0.06% dose (n = 8) ($P < 0.001$). Corrected for the free hormone concentration, the 15-min [^{125}I]Triac uptake in 0.5% BSA was 0.245 ± 0.021 fmol/pM free Triac (n = 8), and that in 0.1% BSA was 0.231 ± 0.008 fmol/pM free Triac (n = 8; P = NS). Therefore, the 15-min uptake experiments were performed in medium with 0.1% BSA.

Figures 3 and 4 show the results of four experiments, in which the competitive effects of various concentrations of unlabeled Triac, T_3, T_4, or Tetrac were tested. The presence of 10 nM Triac resulted in significantly lower uptake of [^{125}I]Triac (35%; $P < 0.001$), whereas 10 μM Triac produced a maximal inhibitory effect of 56% ($P < 0.001$; Fig. 3). A similar inhibitory effect on [^{125}I]Triac uptake was obtained in the presence of 10 μM T_3, T_4, or Tetrac (Fig. 4). When [^{125}I]T_3 uptake was measured in the presence of 10 μM T_3, T_4, Triac, or Tetrac, uptake was reduced by 70%, 60%, 47%, and 48%,

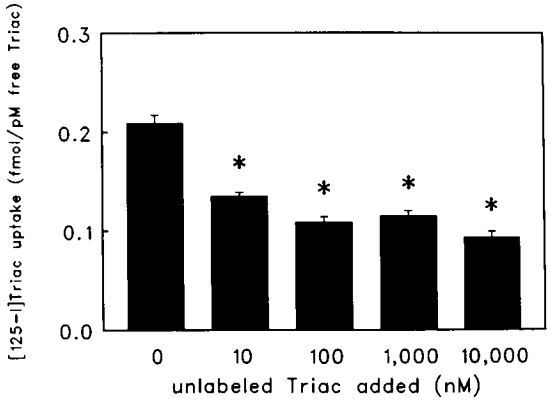

FIG. 3. Effects of increasing concentrations unlabeled Triac on the 15-min uptake of [^{125}I]Triac in cultured anterior pituitary cells. Cells were cultured for 3 days at a density of 500,000 cells/well. After removal of the culture medium, they were preincubated for 30 min in medium containing 0.1% BSA. This was followed by incubation for 15 min in the same medium, with [^{125}I]Triac (100,000 cpm) and unlabeled Triac added at concentrations of 10 nM to 10 μM. *Bars* represent the mean ± SE of six to eight observations from two independent experiments. *, $P < 0.001$ *vs.* no additions.

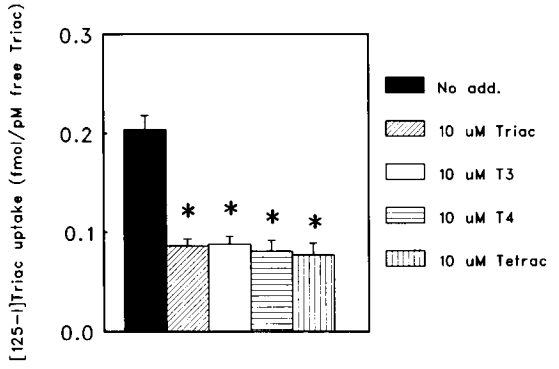

FIG. 4. Effects of Triac, T_3, T_4, and Tetrac on the 15-min uptake of [^{125}I]Triac in cultured anterior pituitary cells. Cells were cultured for 3 days at a density of 500,000 cells/well. Experiments were performed as described in Fig. 3, without or with 10 μM Triac, T_3, T_4, or Tetrac present during the incubation. *Bars* represent the mean ± SE of six to nine observations from three independent experiments. *, $P < 0.001$ *vs.* no additions.

respectively (all $P < 0.001$).

Preincubation and incubation with MDC, oligomycin, and monensin, can be used to study the possible involvement of receptor-mediated endocytosis, energy dependence, and the dependence of the uptake process on the Na^+ gradient, respectively (17, 18). When tested at a concentration of 10 μM, MDC reduced the 15-min uptake of [^{125}I]Triac by 19% (n = 11; $P < 0.005$), oligomycin reduced it by 23% (n = 11; $P < 0.001$), and monensin reduced it by 15% (n = 11; $P < 0.005$; Fig. 5).

Finally, the 15-min uptake of [^{125}I]Triac was directly compared with that of [^{125}I]T_3 with respect to the energy dependence and temperature dependence of the uptake process. Under control conditions, the uptake of [^{125}I]T_3 amounted to 1.55 ± 0.05% dose (n = 9), and that of [^{125}I]Triac was 1.50 ± 0.05% dose (n = 11; Fig. 6, *first and third columns*). Expressed per pM free hormone, the uptake of [^{125}I]T_3 was 0.112 ± 0.004 (n = 9), and that of [^{125}I]Triac was 0.204 ± 0.006 fmol (n = 11; $P < 0.001$). Preincubation and incubation with 10 μM oligomycin reduced the cellular ATP content by 51% ($P < 0.001$; Fig. 6, *last two columns*). At the same time, the uptake of [^{125}I]T_3 was reduced by 77% ($P < 0.001$), and that of [^{125}I]Triac by 25% ($P < 0.001$). On the other hand, the temperature dependences of the two uptake processes were comparable. At 0 C, uptake of [^{125}I]Triac was 0.19 ± 0.04% dose (n = 6), and that of [^{125}I]T_3 was 0.11 ± 0.03% dose (n = 6). Both increased 5-fold when the temperature was increased to 22 C: [^{125}I]Triac uptake, 0.89 ± 0.02% dose (n = 6); [^{125}I]T_3 uptake, 0.80 ± 0.04% dose (n = 6). When the temperature was raised from 22 to 37 C, the uptake of the two isotopes increased 2-fold: [^{125}I]Triac uptake, 1.76 ± 0.06% dose (n = 6); [^{125}I]T_3 uptake, 1.71 ± 0.04% dose (n = 6).

Discussion

The results of the present study demonstrate that [^{125}I]Triac is rapidly taken up by the pituitary. At comparable free medium concentrations (Triac, 0.6–2.2 pM; T_3, 1.7 pM), both cellular and nuclear uptakes of [^{125}I]Triac were at least twice

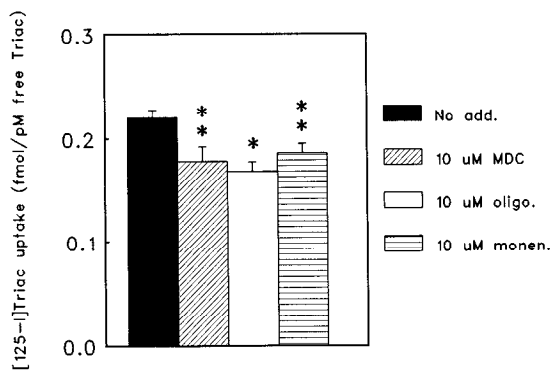

FIG. 5. Effects of MDC, oligomycin, and monensin on the 15-min uptake of [^{125}I]Triac in cultured anterior pituitary cells. Cells were cultured for 3 days at a density of 500,000 cells/well. Preincubation (30 min) and incubation (15 min) were performed as described in Fig. 3, except that MDC, oligomycin, and monensin (10 μM) were present during preincubation and incubation. *Bars* represent the mean ± SE of 11 observations from 4 independent experiments. *, $P < 0.001$; **, $P < 0.005$ (*vs.* no additions).

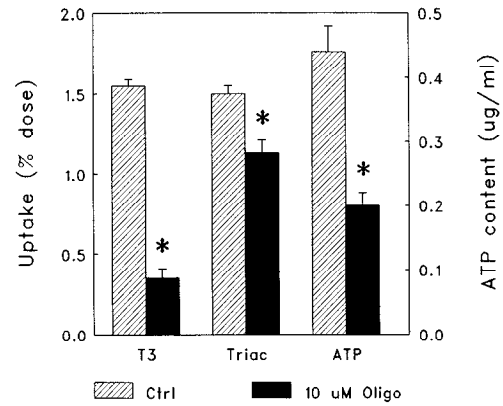

FIG. 6. Effects of oligomycin on the 15-min uptake of [^{125}I]T_3 and [^{125}I]Triac and on ATP contents of cultured anterior pituitary cells. Anterior pituitary cells were cultured for 3 days at a density of 500,000 cells/well. Cells were preincubated for 30 min without or with 10 μM oligomycin. Uptake experiments were performed for 15 min with [^{125}I]T_3 (50,000 cpm/well in medium with 0.5% BSA) or [^{125}I]Triac (100,000 cpm/well in medium with 0.1% BSA) without or with 10 μM oligomycin present. Cells used for determination of ATP content were preincubated and incubated without or with 10 μM oligomycin, but without tracer. *Bars* show the mean ± SE of 9–14 observations from 6 experiments. *, $P < 0.001$ *vs.* controls.

those of [^{125}I]T_3. Furthermore, Triac was, on the basis of the free hormone concentration, more effective than T_3 or T_4 in suppression of basal and TRH-induced TSH release.

These comparisons were made in anterior pituitary cells *in vitro*, *i.e.* an experimental system in which differences in the MCR do not play a role, and neither [^{125}I]Triac nor [^{125}I]T_3 was metabolized. On the other hand, the cells showed metabolic activity, because they were previously shown to deiodinate around 20% of cellular [^{125}I]T_4 to [^{125}I]T_3 in 24 h (18).

In the 2-h incubation experiments with TRH, Triac was, on the basis of the total hormone concentration, equally potent as T_3 in suppression of the TRH-induced TSH release. To obtain a similar effect with T_4, at least a 10-fold higher total dose was required compared to Triac or T_3. However, when the difference in the free fractions of Triac, T_3, and T_4 is taken into account (ratio of the free fractions, 1:8:1), the order of potency for inhibition of TRH-induced TSH release appears to be Triac > T_3 > T_4. A significant reduction of the TSH response to TRH was seen with concentrations as low as 1 nM Triac or T_3. The anterior pituitary cell preparation may be more sensitive to demonstrate these effects than superfused pituitary fragments (16). In the latter system, 100 nM Triac or T_3 (in medium with 0.25% BSA) was required to significantly reduce the TSH response to TRH.

It has been demonstrated *in vivo* that T_3 or T_4 injected into hypothyroid rats reduced the plasma TSH level within 2 h (26). This effect was related to nuclear binding of T_3 (26) and probably reflects interference with the TSH release process. On the other hand, when pituitary cells were exposed for a prolonged period of time (1–3 days during culture) to Triac, T_3, T_4, or TRH, the results were more likely to reflect interference with TSH synthesis. This idea was supported by the finding that the total TSH (medium plus cells) was significantly increased after culture in the presence of 1 nM TRH, suggesting *de novo* TSH synthesis, which confirmed the early

results of Vale *et al.* (27).

Triac seemed to be the most effective in suppression of TSH release during culture. Addition of the same total concentration of T_4 (10 nM) showed a smaller effect on TSH release than that obtained with T_3 or Triac after both 1 and 3 days of culture. When it is taken into account that in medium with 10% serum, the free Triac fraction is much lower than that of T_3, Triac is the most effective in inhibition of TSH synthesis. This was also observed in thyrotropic tumor cells cultured in the presence of serum, when thyroid hormones or analogs were added for 24 h (15). In that study, T_3 and Triac were equally potent when total hormone concentrations were compared, and both showed a 10-fold higher potency than T_4.

Both cellular and nuclear uptakes per pM free hormone of [^{125}I]Triac were twice those of [^{125}I]T_3. Studies with nuclei isolated from thyrotropic tumor cells, pituitary tumor cells, normal rat liver, or heart demonstrated an equal or even higher potency of Triac compared to T_3 to displace nuclear [^{125}I]T_3 binding (15, 28–30), indicating a high affinity for Triac of the thyroid hormone receptor.

The initial rate of [^{125}I]Triac uptake per pM free hormone measured over 15 min was twice that of [^{125}I]T_3. The latter was previously shown to be as high as that of [^{125}I]T_4 (18). The 15-min [^{125}I]Triac uptake was reduced by simultaneous incubation with unlabeled Triac at concentrations as low as 10 nM, suggesting the existence of a carrier in the pituitary cell membrane. Apparently, this carrier also recognized T_3, T_4, and Tetrac, as each of these substances reduced [^{125}I]Triac uptake to the same extent as Triac when added at a concentration of 10 μM.

The experiments with oligomycin showed that the uptake of [^{125}I]Triac was only partially dependent on the cellular energy status; the effect of oligomycin on the uptake of [^{125}I]Triac was only 50% of that on the uptake of [^{125}I]T_3. Furthermore, the experiments with monensin indicate that the uptake process was not strictly dependent on the Na^+ gradient. Compared with our previous observations on the effect of monensin on the uptake of [^{125}I]T_3 or [^{125}I]T_4 (~40%) (17, 18), the effect on the uptake of [^{125}I]Triac was much smaller. The same conclusion holds for the effect of MDC. Part of this difference might be due to the fact that the data were not corrected for nonspecific uptake of [^{125}I]Triac or [^{125}I]T_3, and that the nonspecific uptake of [^{125}I]Triac might have been larger than that of [^{125}I]T_3. On the other hand, the temperature dependence of the uptake of [^{125}I]Triac and [^{125}I]T_3 was the same.

Together, our results suggest that [^{125}I]Triac is taken up by pituitary cells by a carrier-mediated mechanism that is influenced to only a minor extent by the cellular energy status or the Na^+ gradient. [^{125}I]Triac uptake, expressed per pM free hormone was twice as high as that of [^{125}I]T_3. Nevertheless, the uptake mechanism for Triac shared some properties with the transport of T_3 (and T_4). This could mean that Triac is partly taken up by the anterior pituitary by the same transport mechanism as that for T_3 and T_4 and partly by another transport system.

When Triac is used to suppress TSH secretion in patients, it has to be administered in relatively large doses (9–11) due to its short half-life (~6 h) (7). This idea is supported by the observation that dividing the daily Triac dose into four parts had a more pronounced effect on serum TSH in euthyroid subjects than a single dose (8). One of the factors that could explain the short half-life of Triac in humans is the recent finding that Triac is extremely rapidly glucuronidated in the liver (31).

It was recently suggested that Triac might be of importance in the euthyroid sick syndrome (3–5). This syndrome is characterized by low serum T_3 and T_4 levels without a rise in serum TSH (32). The normal serum level of Triac is about 50 pM (2) compared to a normal serum T_3 level of 2000 pM (26). As the free fraction of Triac in serum is much lower than that of T_3 (33), the difference in free hormone concentration in normal subjects may be a factor of 400. During the euthyroid sick syndrome, the serum T_3 level may drop as much as 50%, with a concomitant decrease in the free T_3 concentration (32). In contrast to T_3, Triac is strongly bound to prealbumin in serum, and the prealbumin level decreases substantially during illness (5), leading to a rise in the free Triac fraction (3- to 9-fold) (Everts, M. E., and T. J. Visser, unpublished). This effect should be added to an increased Triac production, whereas deiodination of T_4 to T_3 is diminished (3, 4). Together with our observations that the thyrotroph seems to be more sensitive to Triac than to T_3 and the fact that the uptake of Triac is higher than that of T_3, it is possible that Triac plays a role in the control of TSH secretion in the euthyroid sick syndrome.

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