

Characteristics of 3,5,3'-triiodothyronine (T₃)-uptake system of tadpole red blood cells: effect of endocrine-disrupting chemicals on cellular T₃ response

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Abstract

We characterized the 3,5,3'-L-triiodothyronine (T₃)-uptake system on the plasma membrane of *Rana catesbeiana* tadpole red blood cells (RBCs) in the presence of a variety of inhibitors and potentially competing amino acids. Saturable [¹²⁵I]T₃ uptake was inhibited by phloretin, monodansylcadaverine, bromosulphophthalein, sodium taurocholate and tryptophan. Saturable uptake obeyed simple Michaelis–Menten kinetics with an apparent K_m of 110 nM and a V_{max} of 2.5 pmol/min per 10⁶ cells at 23 °C. These results suggested that a large proportion of T₃ transported into RBCs was mediated by the aromatic amino acid transporter (System T)-linked transporter. To investigate the effect of endocrine-disrupting chemicals (EDCs) on [¹²⁵I]T₃ uptake, RBCs were incubated with [¹²⁵I]T₃ in the presence of each chemical. Among the test chemicals, di-*n*-butyl phthalate, *n*-butylbenzyl phthalate and the miticide, dicofol, were the most powerful inhibi-

tors of [¹²⁵I]T₃ uptake, with an IC₅₀ of 2.2 µM, which was one order of magnitude greater than that for T₃ (IC₅₀, 0.14 µM), and diethylstilbestrol and ethinylestradiol were modest inhibitors. Tributyltin accelerated saturable initial [¹²⁵I]T₃ uptake by 2-fold at 3.2 µM. When RBCs were cultured with 10 nM T₃ at 25 °C for 2 days in the presence of monodansylcadaverine, ethinylestradiol, ioxynil or dicofol at the defined concentrations, these compounds inhibited significantly the induction of the thyroid hormone receptor α gene by T₃. However, not all chemicals competed with T₃ binding to the receptor at the same concentrations. Our results raise the possibility that the T₃-uptake system on the plasma membrane of the tadpole RBCs could be a candidate target site for some EDCs and can modulate cellular T₃ response.

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Introduction

Thyroid hormones (THs), like steroids, retinoids and vitamin D, are hydrophobic signaling molecules. Within the cell, their actions are mediated by nuclear receptors, which regulate the expression of specific target genes. The nuclear receptors for these molecules belong to a superfamily of hormone nuclear receptors, which function as ligand-activated transcription factors (Yen & Chin 1994). 3,5,3'-L-Triiodothyronine (T₃) is the active form of TH that binds to TH nuclear receptors (TRs). Before reaching its intracellular targets, TH must cross the plasma membrane. T₃ either enters the cell directly from plasma or is converted from the precursor form of TH, L-thyroxine (T₄), by deiodinases in the target cells. Because of the lipophilic nature of TH, it was thought that TH traversed the plasma membrane by simple diffusion. However, in the past decade, increasing evidence from a variety of biological sources indicates that TH is actively imported and exported at the cell surface via membrane-bound

transporters. TH transporters characterized so far include amino acid transporters and organic anion transporters (Hennemann *et al.* 2001). The amino acid transporters involved in TH uptake consist of the transporter of large zwitterionic amino acids (System L) and the transporter linked to aromatic amino acid transporters (System T; Blondeau *et al.* 1988), both of which are Na⁺-independent systems. The organic anion transporters comprise a large family of Na⁺-independent transporters and Na⁺-dependent taurocholate transporters (Abe *et al.* 2002). Very recently, it was found that fatty acid translocase, which is a family distinct from the above transporters, can transport TH into cells (van der Putten *et al.* 2003). Experiments involving the overexpression and inactivation of System L demonstrated an important role of this system in the regulation of gene expression mediated by TRs (Ritchie *et al.* 2003). Therefore, TH transport via these transporters may be one of the critical processes controlling the concentration of intracellular TH and, subsequently, the regulation of gene expression mediated by TRs.

Our recent studies have focused on the effects of endocrine-disrupting chemicals (EDCs) on the amphibian and avian thyroid systems, especially on T_3 binding to the major plasma TH-binding protein, transthyretin (TTR), and to TR. These studies revealed that most EDCs tested at micromolar concentrations did not compete with T_3 binding to TR but did compete with T_3 binding to TTR (Yamauchi *et al.* 2000, 2002, 2003, Ishihara *et al.* 2003a, 2003b). This was in contrast to the steroid system where a number of chemicals are capable of competing with estrogen and progesterone binding to their respective receptors (Tran *et al.* 1996, Matthews *et al.* 2000). To understand the action of EDCs in the thyroid system, a research focus on processes other than the competitive binding to TR is required. Recent studies on the effects of agricultural chemicals on the thyroid system support their action on the processes other than the competitive inhibition of T_3 binding to TRs. The herbicide acetochlor, for which amphibian TRs and TTRs had no significant affinity (Yamauchi *et al.* 2002, Ishihara *et al.* 2003a), altered TH-dependent gene expression and amphibian metamorphosis (Cheek *et al.* 1999, Veldhoen & Helbing 2001, Crump *et al.* 2002). *Xenopus* tadpoles previously exposed to methoprene did not respond to exogenously added T_4 (Fort *et al.* 2000), although *Xenopus* TR and TTR showed no significant affinity for this chemical (Yamauchi *et al.* 2002). However, there have been few reports that focus on processes other than competitive binding of EDCs to TTR and TR in the thyroid system.

To elucidate the role of a T_3 -uptake system on the cellular T_3 response and identify what kind of EDCs target the T_3 -uptake system and interfere with the thyroid system, we investigated the characteristics of T_3 uptake into *Rana catesbeiana* tadpole red blood cells (RBCs). We chose 35 chemicals: 11 chemicals that inhibit the activity of TH transporters (Blondeau *et al.* 1988, McLeese & Eales 1996a) and 24 chemicals known to affect plasma TH levels in mammals (Brucker-Davis 1998), and examined their effects on [125 I] T_3 uptake into the tadpole RBCs. In this study, it was found that T_3 was transported into tadpole RBCs by the System T-linked transporter. Several compounds, including EDCs, significantly inhibited [125 I] T_3 uptake in a dose-dependent manner. Furthermore, some of these inhibitors of the T_3 -uptake system suppressed the induction of the early primary T_3 -response gene *tra* by T_3 . We report here that the T_3 -uptake system on the plasma membrane of tadpole RBCs can modulate T_3 signaling by controlling the amount of intracellular T_3 , and that it could be a candidate target site for some EDCs that affect the thyroid system.

Materials and Methods

Reagents

[125 I] T_3 (122 MBq/ μ g; carrier-free) was purchased from NEN Life Science Products (Boston, MA, USA). Un-

labeled T_3 , bisphenol A, 1-aminopyrene, benzo[*a*]pyrene, pentachlorophenol, 2-amino[2,2,1]heptane-2-carboxylic acid (BCH), monodansylcadaverine and ouabain were obtained from Sigma (St Louis, MO, USA). Ethinyl-estradiol, dicyclohexyl phthalate, tributyltin chloride, benzophenone, bis(2-ethylhexyl) adipate, *p*-*t*-octylphenol, mirex (dodecachloropentacyclo [5·3·0·0^{2,6}·0^{3,9}·0^{4,8}]*decane*, analytical standard), malathion (diethyl(dimethoxythio-phosphorylthio)succinate, analytical standard), dicofol (Kelthane; 2,2,2-trichloro-1,1-bis (4-chlorophenyl)ethanol, analytical standard), *L*-tryptophan, *L*-phenylalanine, *L*-leucine, *N*-ethylmaleimide, phloretin, sodium taurocholate and choline chloride were purchased from Wako Pure Chemicals (Osaka, Japan). Ioxynil (3,5-di-iodo-4-hydroxybenzonitril, analytical standard) and acetochlor (2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide) were purchased from Riedel-de Haën (Seeize, Germany). Methoprene ((*E,E'*)-1-methylethyl-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate (9 Cl)) was from Ehrenstorfer Quality (Augsburg, Germany). 4-Nonylphenol, di-*n*-butyl phthalate and *n*-butylbenzyl phthalate were obtained from Nacalai Tesque (Kyoto, Japan). 2,4-Dichlorophenoxyacetic acid, di-2-ethylhexyl phthalate, diethyl phthalate and 2,4-dinitrophenol were from Kanto Chemicals (Tokyo, Japan), and diethylstilbestrol (DES) was from Tokyo Chemical Industry (Tokyo, Japan). Bromosulfophthalein was purchased from ICN Biochemicals (Irvine, CA, USA). All other chemicals used in this study were of chromatography grade or the highest grade available and purchased from Wako Pure Chemicals or Nacalai Tesque.

All chemicals tested as EDCs, except for 1-aminopyrene, benzo[*a*]pyrene and mirex, which were dissolved in benzene to a concentration of 10 mM, were dissolved in ethanol to concentrations of 5–10 mM. Phloretin and T_3 were dissolved in dimethylsulfoxide to concentrations of 0.6–200 mM. Monodansylcadaverine was dissolved in methanol to a concentration of 50 mM. Tryptophan, leucine, phenylalanine, bromosulfophthalein, BCH, sodium taurocholate, ouabain, *N*-ethylmaleimide and choline chloride were dissolved in frog Ringer solution (111 mM NaCl, 3.4 mM KCl, 2 mM $CaCl_2$ and 2.4 mM $NaHCO_3$) or 70% Leibovitz's L-15 medium (Sigma). These chemicals were diluted with the frog Ringer solution or 70% Leibovitz's L-15 medium to give less than 0.5% (v/v) organic solvent. Control assays were performed in the presence of the corresponding solvent only and at the same concentrations. The organic solvents added did not affect the assays for T_3 uptake, binding or cellular response.

Preparations of RBCs

R. catesbeiana tadpoles at stages X–XV (Taylor and Kallros 1946) were collected from ponds in Saitama Prefecture and Shizuoka Prefecture, Japan. The tadpoles were

maintained in aerated, dechlorinated tap water at 20–25 °C, unless otherwise noted, and fed boiled spinach three times a week. They were anesthetized by immersion in 0.2% ethyl 3-aminobenzoate methanesulfonic acid (Sigma). RBCs were prepared as described previously (Yamauchi *et al.* 1989). In brief, the truncus arteries of the tadpoles were cut with scissors and blood samples were collected into tubes containing heparin or sodium citrate. The plasma was separated from the blood cells by centrifugation at 1500 g for 15 min at 4 °C. After removal of the plasma and the buffy coat, the RBC fraction was further washed at least seven times with the frog Ringer solution or 70% Leibovitz's L-15 medium. The number of RBCs was estimated by counting under a microscope with the use of a hemacytometer. The RBC suspension was kept on ice and used within a day. For 2-day experiments, the RBC suspension was used at 25 °C immediately after preparation.

Uptake of [¹²⁵I]T₃ into tadpole RBCs

Tadpole RBCs were incubated in the frog Ringer solution or 70% Leibovitz's L-15 medium at 23 °C. Uptake was initiated by mixing the RBC suspension (1.0 × 10⁶ cells/tube) with [¹²⁵I]T₃ solution at the same temperature, adjusting the final concentration of [¹²⁵I]T₃ to 0.1 nM in the presence or absence of 5 μM unlabeled T₃. To examine the effect of chemicals, the RBC suspension was mixed with [¹²⁵I]T₃ solution containing each chemical at 23 °C without pre-incubation. After 2 min incubation at 23 °C, the cell-associated [¹²⁵I]T₃ and free [¹²⁵I]T₃ were separated by the oil-centrifugation method (Yamauchi *et al.* 1989, 2000). The cells were immediately separated from free [¹²⁵I]T₃ in the incubation medium by centrifugation at 9100 g for 2 min. The tip of the tube containing the cell pellet was cut off. The amount of [¹²⁵I]T₃ associated with cells and the amount of [¹²⁵I]T₃ remaining in the extracellular fraction were determined in a gamma scintillation counter (Auto Well Gamma System ARC-2000, Aloka, Japan).

Kinetic studies were carried out at 23 °C for 2 min. Kinetic parameters were determined by fitting the plot of initial velocity (*V*_i) versus T₃ concentration (*S*) to the Michaelis–Menten equation: $V_i = V_{max}/(1 + K_m/S)$, where *V*_{max} is the maximum uptake rate and *K*_m is the Michaelis constant.

RNA analysis by real-time PCR

Tadpole RBCs (5.0 × 10⁶ cells/35 mm dish) were incubated in 70% of Leibovitz's L-15 medium for 2 days at 25 °C with solvent only or each chemical in the presence or absence of 10 nM T₃. The medium did not change during the incubation. Total RNA was extracted from tadpole RBCs using a QIAamp RNA Blood Mini kit (Qiagen, Hilden, Germany) according to the

manufacturer's directions. The RNA (5 μg per lane) was electrophoresed in 1% agarose gel containing 2.6 M formaldehyde to check the integrity of the RNA samples. Amounts of specific RNA species were estimated by real-time PCR using SYBR Green Master Mix and an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA, USA) after the RNA samples were treated with reverse transcriptase (TaqMan Reverse Transcription Reagents; Applied Biosystems). Each PCR was run in triplicate to control for PCR variation. The endpoint used in real-time PCR quantification, Ct, is defined as the PCR cycle number that crosses an arbitrarily placed signal threshold and is a function of the amount of target DNA present in the starting material. Quantification was determined by applying the 2^{-Ct} formula and calculating the average of the three values obtained for each sample. To standardize each experiment, the amount of bullfrog TRα (bTRα) transcript was divided by the amount of 18 S rRNA in the same samples. Primer sequences used were as follows: bTRα transcript sense 5'-GCGTCGGAAGGA GGAGATG-3' (nucleotides 229–247) and antisense 5'-TCCCCTCCTCGCTGCTT-3' (294–277; Schneider & Galton 1991); and 18 S rRNA sense 5'-TGTGC CGCTAGAGGTGAAATT-3' (908–928) and antisense 5'-TGGCAAATGCTTTCGCTTT-3' (970–952), which were designed by comparing the corresponding *Xenopus laevis* and human sequences (Maden 1986).

[¹²⁵I]T₃-binding assay

The ligand-binding domain of bTRα fused with glutathione *S*-transferase (GST-bTRα LBD) was expressed in *Escherichia coli* and then purified from the cell extract as described previously (Ishihara *et al.* 2003a). Competitive [¹²⁵I]T₃ binding was performed with solvent only or each chemical, which was diluted with a buffer (10 mM Tris/HCl, pH 7.5, 1.5 mM EDTA, 1 mM dithiothreitol and 10% (v/v) glycerol) containing [¹²⁵I]T₃, at defined concentrations. [¹²⁵I]T₃ binding was initiated by mixing 50 μl GST-bTRα LBD (150 ng/tube) in the buffer with 200 μl of the above buffer containing [¹²⁵I]T₃ with or without each chemical at 4 °C, adjusting the final concentration of [¹²⁵I]T₃ to 0.1 nM. After 1.5 h incubation at 4 °C, protein-bound [¹²⁵I]T₃ was separated from free [¹²⁵I]T₃ by the Dowex method (Lennon *et al.* 1980, Lennon 1992). Radioactivity was measured in a gamma scintillation counter. The amount of [¹²⁵I]T₃ bound non-specifically was derived from the radioactivity of the samples incubated with 1 μM unlabeled T₃. The non-specific binding value was subtracted from the amount of total bound T₃ to give the value of specifically bound [¹²⁵I]T₃.

Statistical analysis

The data are presented as means ± S.E.M. Differences between groups were analyzed with either Student's *t* test

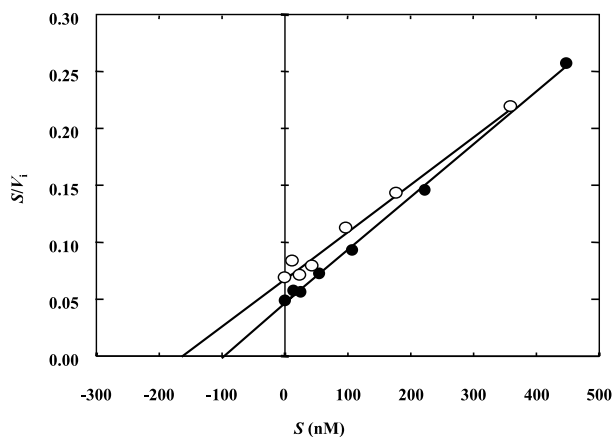


Figure 1 Hanes plots of [^{125}I] T_3 uptake into bullfrog tadpole RBCs. Tadpole RBCs were incubated for 2 min at 23 °C in 70% Leibovitz's L-15 medium (○) or the frog Ringer solution (●) containing 0.1 nM [^{125}I] T_3 and various concentrations (S) of unlabeled T_3 to determine initial velocity (V_i). Values are means of triplicates. Kinetic constants were obtained by linear regression. The abscissa intercepts of the lines correspond to $-K_m$, the ordinate intercepts to K_m/V_{\max} and the slopes to $1/V_{\max}$. The graphs were drawn after subtraction of nonsaturable uptake, as described in the Materials and Methods section.

or Cochran–Cox test to evaluate the significance of the differences. $P < 0.05$ was considered statistically significant. The number of observations (n) is shown in parentheses.

Results

Characteristics of T_3 -uptake system on the plasma membrane of tadpole RBCs

[^{125}I] T_3 uptake into tadpole RBCs reached equilibrium by 10 min at 23 °C. As the initial uptake was approxi-

mately linear for at least the first 3-min period, the V_i of uptake was routinely measured after 2 min of incubation. Saturability of T_3 uptake was demonstrated by measuring [^{125}I] T_3 uptake in the presence of 5 μM unlabeled T_3 . Approximately 85% of the V_i of [^{125}I] T_3 uptake was inhibited by 5 μM unlabeled T_3 (data not shown). These experiments suggest that a saturable uptake system is responsible for transporting most of the circulating T_3 into RBCs.

Saturable uptake conformed to Michaelis–Menten kinetics. RBCs were incubated with 0.1 nM [^{125}I] T_3 and various concentrations of unlabeled T_3 (S, ranging from 10^{-8} to 10^{-6} M). The ratio of S/V_i was calculated and plotted as a function of S according to the Hanes representation (Fig. 1). The plots are linear and repeated experiments gave a K_m of 110 ± 10 nM and a V_{\max} of 2.5 ± 0.3 pmol/min per 10^6 cells in the frog Ringer solution ($n=3$) and a K_m of 170 ± 20 nM and a V_{\max} of 2.3 ± 0.2 pmol/min per 10^6 cells in 70% Leibovitz's L-15 medium ($n=5$). The K_m value was 1.5 times greater when 70% Leibovitz's L-15 medium was used instead of the frog Ringer solution. It is believed that this culture medium may have contained some competitors that interfered with the saturable T_3 -uptake system of tadpole RBCs.

To characterize the saturable T_3 -uptake system in tadpole RBCs, [^{125}I] T_3 uptake was investigated in the presence of chemicals known to inhibit the activity of amino acid transporters (Table 1). Of the three amino acids tested, tryptophan was the most powerful inhibitor while phenylalanine was a weak inhibitor and leucine had no effect, at 1 mM. The effect of BCH, a prototypical substrate of the amino acid transporter System L, was weak even at 5 mM. Organic anions, bromosulphophthalein and taurocolate were strong inhibitors. Bromosulphophthalein, at the concentration of 0.3 mM, inhibited [^{125}I] T_3 uptake by more than 90%. Choline chloride (111 mM), substituted

Table 1 Effect of compounds known to inhibit the activity of amino acid transporters on [^{125}I] T_3 uptake into tadpole RBCs. Each value shown is the mean \pm S.E.M.

Inhibitor	Concentration (mM)	[^{125}I] T_3 uptake (% of control)	n
Control (frog Ringer solution)		100 ± 3	7
Tryptophan	1	$24 \pm 3^*$	7
Leucine	1	94 ± 3	7
Phenylalanine	1	$76 \pm 3^*$	7
BCH	5	$73 \pm 4^*$	4
Bromosulphophthalein	0.3	$9 \pm 2^*$	3
Sodium taurocolate	0.5	$45 \pm 4^*$	4
Choline chloride	111	108 ± 1	3
N-Ethylmaleimide	1	$79 \pm 5^*$	4
Ouabain	1	91 ± 2	4
Control (dimethylsulfoxide)		100 ± 6	4
Phloretin	0.1	$22 \pm 2^*$	4
Control (methanol)		100 ± 6	4
Monodansylcadaverine	0.5	$22 \pm 3^*$	4

* $P < 0.001$, significant difference from the control values.

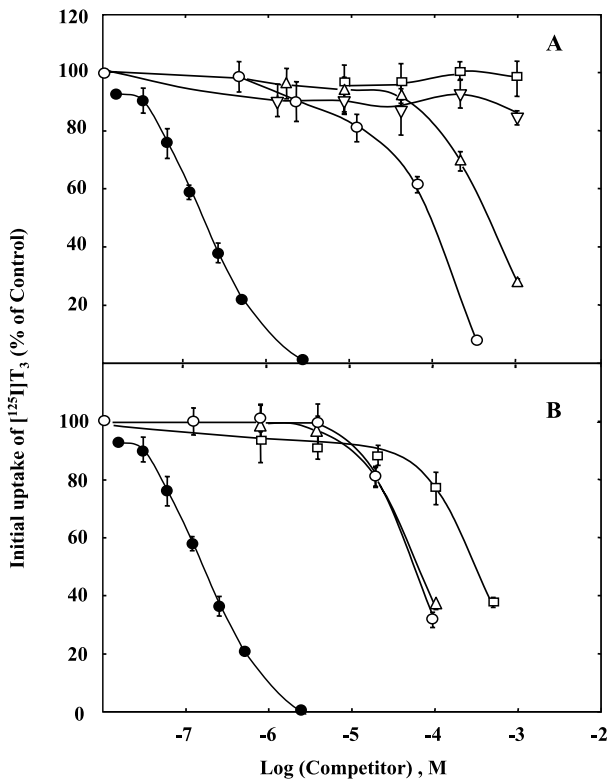


Figure 2 Dose-dependent inhibition of [¹²⁵I]T₃ uptake into tadpole RBCs by inhibitors of amino acid transporters. Saturable initial uptake of [¹²⁵I]T₃ into tadpole RBCs was examined in the frog Ringer solution in the presence or absence (control) of various concentrations of each inhibitor of amino acid transporters: bromosulphophthalein (O) tryptophan (Δ), leucine (∇) or BCH (□) in (A), and phloretin (O), monodansylcadaverine (Δ) or taurocholate (□) in (B), as described in the Materials and Methods section. ● show the effect of unlabeled T₃ on [¹²⁵I]T₃ uptake in each panel. Each point represents the mean ± S.E.M. from triplicate determinations. Deviations less than the size of the symbols are not shown. These experiments were repeated at least three times.

for sodium chloride in the frog Ringer solution, showed no effect. *N*-Ethylmaleimide (1 mM), a thiol reagent, inhibited only 20% of the saturable T₃ uptake. Ouabain (1 mM) did not inhibit, but phloretin (0.1 mM) and monodansylcadaverine (0.5 mM) inhibited the saturable T₃ uptake by 80%.

Figure 2 shows dose-dependent curves of eight selected compounds, including unlabeled T₃, on the saturable initial uptake of [¹²⁵I]T₃ into tadpole RBCs in the frog Ringer solution. The most effective compound was unlabeled T₃. The rank order affinity was T₃ (the concentration of T₃ required for 50% inhibition of saturable [¹²⁵I]T₃ (IC₅₀)=0.14 μM)>phloretin (52 μM)=monodansylcadaverine (59 μM)>bromosulphophthalein (75 μM)>taurocholate (270 μM)>tryptophan (470 μM)>>leucine=BCH (Table 2). Leucine and BCH had no effect at all concentrations tested.

Effect of EDCs on T₃ uptake into tadpole RBCs

Saturable initial uptake of [¹²⁵I]T₃ into the tadpole RBCs was examined in the presence of various chemicals (8 μM) at 23 °C (Fig. 3). As unlabeled T₃ inhibited completely the saturable initial uptake of [¹²⁵I]T₃ at 5–8 μM, a concentration of 8 μM for the chemicals was chosen to determine their relative potencies. T₄ inhibited the saturable initial uptake of [¹²⁵I]T₃ by 60%, suggesting that this uptake system can recognize both T₃ and T₄ but is more specific for T₃ than for T₄. Of the five xenoestrogens, ethinylestradiol, DES, *p*-*t*-octylphenol, 4-nonylphenol and bisphenol A, ethinylestradiol and DES were the most potent inhibitors: approximately 50% of the saturable initial uptake of [¹²⁵I]T₃ was inhibited. Of the two pyrene compounds, benzo[*a*]pyrene activated the uptake up to 150%. Among the five phthalates, di-*n*-butyl phthalate and *n*-butylbenzyl phthalate were the most potent

Table 2 Inhibition of [¹²⁵I]T₃ uptake into tadpole RBCs by amino acid transport inhibitors. Each value shown is the mean ± S.E.M.

Chemical	IC ₅₀ (μM)	n
Unlabeled T ₃ (in frog Ringer solution)	0.14 ± 0.01	3
Phloretin	52 ± 3	3
Monodansylcadaverine	59 ± 2	3
BSP	75 ± 16	3
Sodium taurocholate	270 ± 30	3
Tryptophan	470 ± 40	5
Leucine	>>1000	3
BCH	>>1000	3
Unlabeled T ₃ (in Leibovitz's L-15 medium)	0.24 ± 0.02	4
Di- <i>n</i> -butyl phthalate	2.2 ± 0.2	3
<i>n</i> -Butylbenzyl phthalate	2.2 ± 0.1	3
Dicofol	2.2 ± 0.2	5
Tributyltin chloride	3.2 ± 0.5*	3

*This value is the ED₂₀₀, because only tributyltin chloride accelerated saturable initial uptake of [¹²⁵I]T₃.

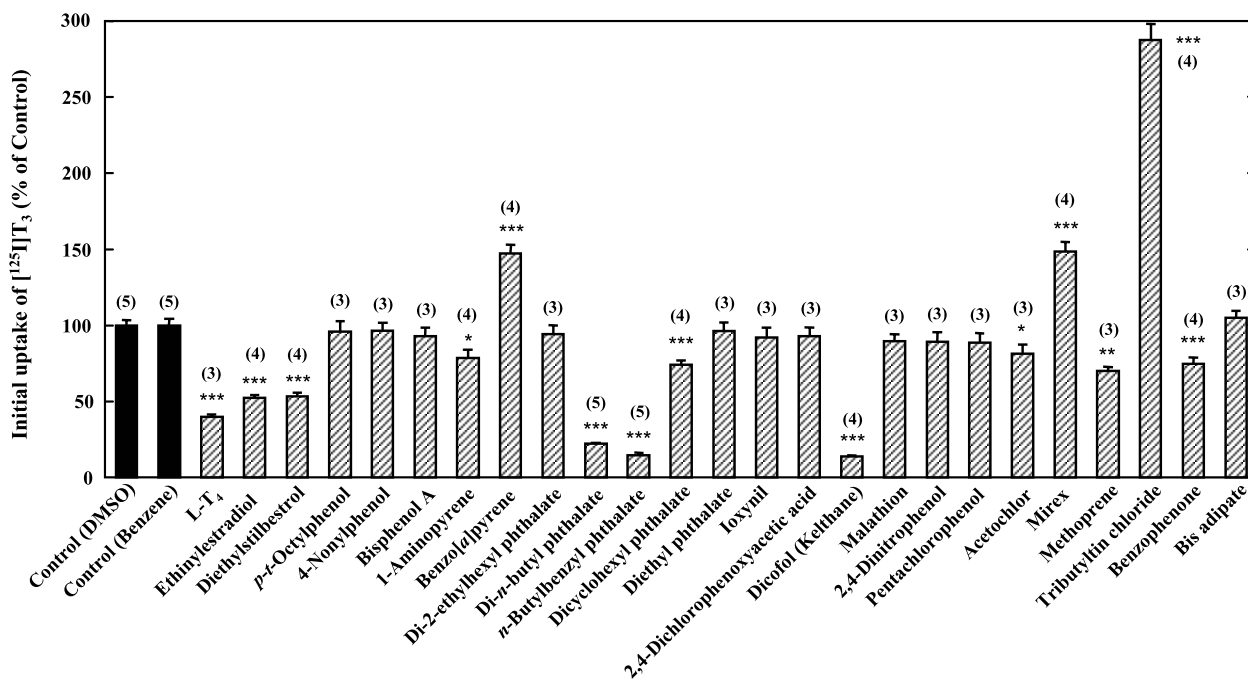


Figure 3 Inhibition of [¹²⁵I]T₃ uptake into tadpole RBCs by chemicals including EDCs. The saturable initial uptake of [¹²⁵I]T₃ into tadpole RBCs was examined in 70% Leibovitz's L-15 medium in the presence (8 μM) or absence (control) of each chemical: thyroxine, five xenoestrogens, two pyrene compounds, five phthalates, eight agricultural chemicals and four other chemicals, from left to right, as described in the Materials and Methods section. The numbers of repeated experiments are shown in parentheses. Each point represents the mean ± S.E.M. from triplicate determinations. These experiments were repeated at least three times. **P*<0.05, ***P*<0.01 and ****P*<0.001, significantly different from control.

inhibitors: approximately 70–80% of the saturable initial uptake of [¹²⁵I]T₃ was inhibited. Of the other chemicals, including agricultural chemicals, dicofof was the most powerful inhibitor: more than 80% of the saturable initial uptake of [¹²⁵I]T₃ was inhibited. Mirex and tributyltin accelerated the saturable initial uptake of [¹²⁵I]T₃ up to 150 and 290%, respectively.

Figure 4 shows dose-dependent curves of the five selected chemicals, including unlabeled T₃, on the saturable initial uptake of [¹²⁵I]T₃ into tadpole RBCs in 70% Leibovitz's L-15 medium. The IC₅₀ values of di-*n*-butyl phthalate, *n*-butylbenzyl phthalate and dicofof were all 2.2 μM (Table 2). The concentration of tributyltin required to accelerate the saturable initial uptake of [¹²⁵I]T₃ up to 200% (ED₂₀₀) was 3.2 μM (Table 2). These effective concentrations were one order of magnitude greater than the IC₅₀ of T₃ (0.24 μM), but may be less than the IC₅₀ of T₄ (approximately 8 μM; see Fig. 3) although we did not determine the accurate IC₅₀ value of T₄.

Effect of the inhibitors of the saturable T₃-uptake system on the induction of TRα transcript by T₃ in tadpole RBCs

To evaluate the biological effect of the inhibitors of the saturable T₃-uptake system on T₃ signaling in tadpole

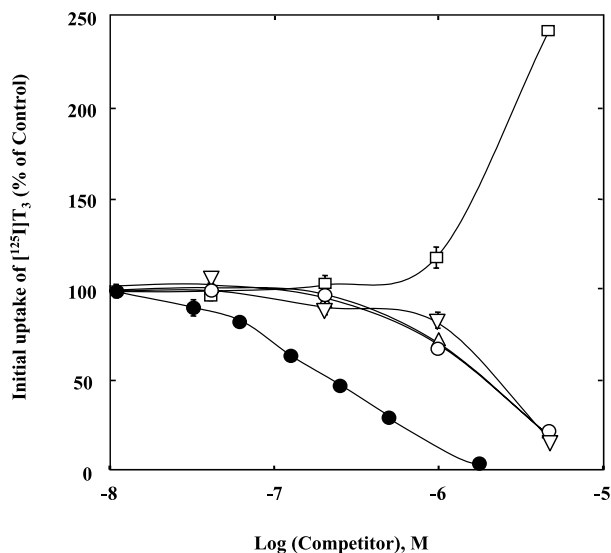


Figure 4 Dose-dependent inhibition of [¹²⁵I]T₃ uptake into tadpole RBCs by EDCs. Saturable initial uptake of [¹²⁵I]T₃ into tadpole RBCs was examined in 70% Leibovitz's L-15 medium in the presence or absence (control) of various concentrations of each EDC: di-*n*-butyl phthalate (○), *n*-butylbenzyl phthalate (△), dicofof (□) or tributyltin chloride (▽), as described in the Materials and Methods section. ● show the effect of unlabeled T₃ on [¹²⁵I]T₃ uptake. Each point represents the mean ± S.E.M. from triplicate determinations. Deviations less than the size of the symbols are not shown. These experiments were repeated at least three times.

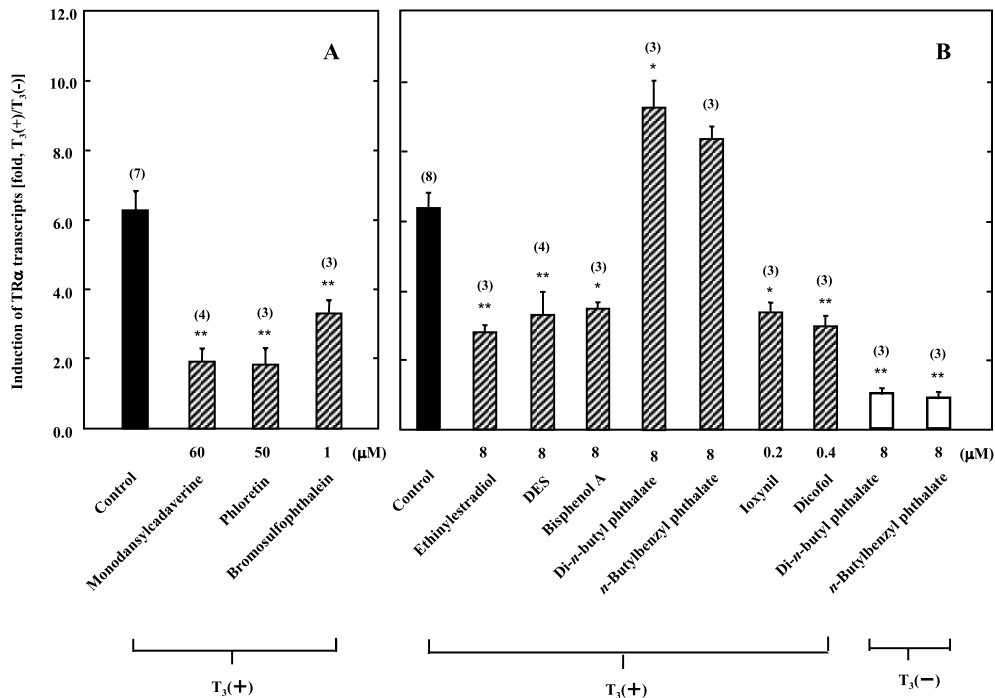


Figure 5 Effect of chemicals on the induction of the early primary T₃-response gene *tra* by T₃ in tadpole RBCs. Induction of the early primary T₃-response gene, *tra*, was examined as an indicator of cellular T₃ response. RBCs were cultured for 48 h at 25 °C in 70% Leibovitz's L-15 medium with or without 10 nM T₃ (control). Some cells were cultured in the presence of both T₃ and each chemical. Chemicals used were three inhibitors of the T₃-uptake system (A) and seven EDCs (B), at the indicated concentrations. Total RNA was prepared from the cultured RBCs. TRα transcript and 18 S rRNA were analyzed by real-time RCR following their reverse transcription, as described in the Materials and Methods section. Amounts of TRα transcript were normalized by those of 18 S rRNA of the same RNA samples. The numbers of repeated experiments are shown in parentheses. Each value is the mean ± S.E.M. *P<0.01 and **P<0.001, significantly different from control.

RBCs, we examined their interference with the induction of the early primary T₃-response gene, *tra* gene, by 10 nM T₃. The addition of T₃ increased the amount of the TRα transcript by 6–7 fold. Further addition of monodansylcadaverine (final concentration of 60 μM), phloretin (50 μM), bromosulphthalein (1 μM), DES (8 μM), ethinylestradiol (8 μM) and dicofol (0.4 μM), depressed significantly the T₃ response (Fig. 5). Di-*n*-butyl phthalate, but not *n*-butylbenzyl phthalate, had a significant synergic effect on the induction of the TRα transcript by T₃ at 8 μM, the concentration at which the saturable initial uptake of [¹²⁵I]T₃ was strongly inhibited by these phthalates (Fig. 3). This effect was abolished in the absence of T₃. Bisphenol A (8 μM) and ioxynil (0.2 μM) inhibited the induction of the TRα transcript by T₃ although they did not inhibit the saturable initial uptake of [¹²⁵I]T₃ at the same or higher concentrations (Fig. 3). We could not evaluate the effect of tributyltin on the induction of the TRα transcript by T₃ because of its severe cytotoxicity at 10⁻⁷–10⁻⁵ M.

To examine whether the chemicals that affected the induction of the TRα transcript by T₃ interact directly

with TRα to compete with T₃ binding, we next examined their effect (at the same concentrations) on [¹²⁵I]T₃ binding to purified GST-bTRα LBD (Fig. 6). Phloretin, bromosulphthalein and DES inhibited [¹²⁵I]T₃ binding to GST-bTRα LBD; however, monodansylcadaverine, ethinylestradiol, bisphenol A, dibutyl phthalate, ioxynil and dicofol showed no effect on [¹²⁵I]T₃ binding to the GST-bTRα LBD at concentrations that affected the induction of the TRα transcript by T₃. The T₃-binding activity of GST was negligible when purified GST, which was expressed from pGEX-6P-3 in *E. coli*, was substituted for the GST-bTRα LBD fusion protein in the assay.

Discussion

Here we have described the inhibitory mode of the T₃-uptake system of bullfrog tadpole RBCs: sensitivity to monodansylcadaverine, phloretin and tryptophan but insensitivity to leucine, BCH and ouabain (Table 1). These were consistent with the inhibitory mode of the

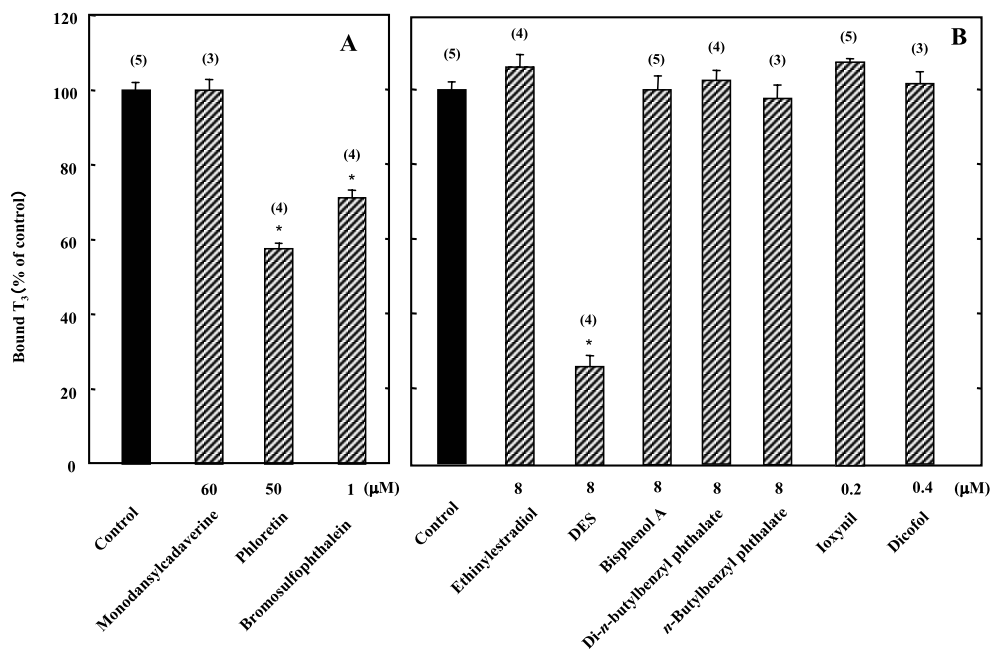


Figure 6 Effect of chemicals on [¹²⁵I]T₃ binding to GST-bTR α LBD. Purified GST-bTR α LBD was incubated with 0.1 nM [¹²⁵I]T₃ for 1.5 h at 4 °C in the presence or absence (control) of each chemical, as described in the Materials and Methods section. Chemicals used were three inhibitors of the T₃-uptake system (A) and seven EDCs (B), at the indicated concentrations. The numbers of repeated experiments are shown in parentheses. Each point represents the mean \pm S.E.M. from triplicate determinations. **P* < 0.001, significantly different from control.

T₃-uptake system of rat and rainbow trout RBCs (Zhou *et al.* 1990, McLeese & Eales 1996a) and rat liver (Blondeau *et al.* 1988), some of which were characterized as System T-linked T₃-uptake system (Osty *et al.* 1988, Samson *et al.* 1992). The fact that the T₃-uptake system in tadpole RBCs was sensitive to taurocholate and bromosulphthalein but was insensitive to choline suggests the possibility that some of Na⁺-independent organic anion transporters (Abe *et al.* 1998, 1999, Fujiwara *et al.* 2001) are involved in the tadpole T₃-uptake system. The *K_m* values obtained from our studies were within the same range of those values obtained for the System T-linked T₃-uptake system (Blondeau *et al.* 1988, Osty *et al.* 1988, Zhou *et al.* 1990, McLeese & Eales 1996b), but one order of magnitude lower than those values obtained for System L (Blondeau *et al.* 1993, Ritchie *et al.* 1999, Ritchie & Taylor 2001, Friesema *et al.* 2001) and the organic anion transporters (rat *oatp2* and *oatp3*, human LST-1 and OATP-E; Abe *et al.* 1998, 1999, Fujiwara *et al.* 2001). Therefore, the T₃-uptake system that is responsible for a major part of saturable T₃ uptake into tadpole RBCs may be the System T-linked uptake system and may be a common feature for rat, bullfrog and rainbow trout RBCs. This is the first report demonstrating the existence of the System T-linked T₃-uptake system in frogs. The expression of this T₃-uptake system in RBCs has probably been conserved during vertebrate evolution. Tadpole RBCs

(larval type) are specifically removed from the bloodstream during metamorphic climax stages by apoptotic cell death under the influence of THs (Hasebe *et al.* 1998). Therefore, the system T-linked T₃-uptake system on the plasma membrane would play an important role in T₃-induced apoptosis in tadpole RBCs. The *K_m* value determined in 70% Leibovitz's L-15 medium was 1.6 times greater than that determined in the frog Ringer solution. As Leibovitz's L-15 medium contains 1.5 mM phenylalanine and 0.1 mM tryptophan, it is likely that these amino acids compete with saturable T₃ uptake into tadpole RBCs.

The effect of *N*-ethylmaleimide on System T-linked T₃-uptake systems was contradictory. *N*-Ethylmaleimide, at a concentration of 1 mM, hardly inhibited saturable [¹²⁵I]T₃ uptake into tadpole RBCs (Table 1). Our result was consistent with the report for rat hepatocytes (Blondeau *et al.* 1988). Like us, Blondeau *et al.* examined [¹²⁵I]T₃ uptake in the presence of *N*-ethylmaleimide without preincubation with it. The saturable [¹²⁵I]T₃ uptake into rat and rainbow trout RBCs was strongly inhibited by *N*-ethylmaleimide when the cells were pretreated with it at 0.5–1.0 mM for 10–15 min (Zhou *et al.* 1990, McLeese & Eales 1996a). However, Kemp & Taylor (1997) found that approximately 40% of the saturable [¹²⁵I]T₃ uptake into sinusoidal membrane vesicles obtained from rat liver homogenate, corresponding to the System T-linked uptake system, was

Table 3 Summary of the possible target sites of chemicals affected on the thyroid system and their effective concentrations. Data were collected from Figs 2–6 and summarized

Chemical	Concentration (μM)	Uptake system	T ₃ binding to TRα	Induction of TRα transcript by T ₃
Phloretin	50	↓	↓	↓
DES	8	↓	↓	↓
Monodansylcadaverine	60	↓	—	↓
Ethinylestradiol	8	↓	—	↓
Bromosulphophthalein	1	—	↓	↓
Ioxynil	0.2	—**	—	↓
Dicofol	0.4	—	—	↓
Bisphenol A	8	—	—	↓
Di- <i>n</i> -butyl phthalate	8	↓	—	↑*
<i>n</i> -Butylbenzyl phthalate	8	↓	—	—***

—, No effect; ↓, inhibition; ↑, activation (*synergic effect with T₃ but no effect without T₃); **, no effect at even 8 μM; ***, no significant effect although this mean value was higher than the mean value of the control.

N-ethylmaleimide-resistant after preincubation with 1 mM *N*-ethylmaleimide even for 0.5 min. cDNA for Na⁺-independent aromatic amino acid transporter was cloned from rat small intestine and its nucleotides were sequenced. However, T₃ and T₄ did not exhibit significant effects on this transporter-mediated [¹⁴C]tryptophan uptake, and the transporter hardly accelerated uptake of [¹²⁵I]T₃ and [¹²⁵I]T₄ into cells (Kim *et al.* 2001). Very recently, Friesema *et al.* (2003) cloned a cDNA for System T-related transporter from rat liver and characterized it. This transporter, termed monocarboxylate transporter 8, induced [¹²⁵I]T₃ uptake in a *Xenopus* oocyte expression system with a K_m of 4 μM, but did not transport tryptophan and phenylalanine. Therefore, several types of System T- and/or System T-linked T₃ uptake system may exist.

We found that di-*n*-butyl phthalate, *n*-butylbenzyl phthalate, dicofol and tributyltin, when added to 70% Leibovitz's L-15 medium, affected the V_i of the saturable [¹²⁵I]T₃ uptake with IC₅₀ (or ED₂₀₀ for tributyltin) of 2.2–3.2 μM (Fig. 4 and Table 2). Tributyltin accelerated the initial uptake of [¹²⁵I]T₃ into tadpole RBCs at 10⁻⁶ M in a 2-min treatment; however, it showed severe cytotoxicity with hemolysis at 10⁻⁷–10⁻⁵ M in a 2-day treatment. Therefore, it is doubtful that tributyltin exerts EDC activity mediated by the T₃-uptake system at these concentrations *in vivo*. These phthalates have been produced abundantly as plasticizers. Dicofol is a chlorinated miticide, which has been applied at high doses to defined agricultural land. Tributyltin has been used in antifouling paints. All of these chemicals are distributed at high concentrations in their special environments. Our results propose a novel mechanism that some chemicals interfere with the thyroid system owing to inhibition or activation of the activity of the T₃-uptake system on the plasma membrane. However, from the above result alone, we cannot simply

explain to what degree the suppression or acceleration of saturable [¹²⁵I]T₃ uptake influences the T₃-signaling pathway in target cells.

To facilitate our understanding of the effect of the chemicals used in this study on TH signaling in tadpole RBCs, their possible target sites are summarized in Table 3, where the chemicals are divided into five groups according to their mode of action. The first group includes the chemicals that inhibited both the T₃-uptake system and T₃ binding to TR: phloretin and DES. The second group consists of the chemicals that inhibited the T₃-uptake system but not T₃ binding to TR: monodansylcadaverine and ethinylestradiol. Conversely, the chemical belonging to the third group, bromosulphophthalein, did not inhibit the T₃-uptake system but did inhibit T₃ binding to TR. The fourth group includes the chemicals that inhibited neither the T₃-uptake system nor T₃ binding to TR: ioxynil, dicofol and bisphenol A. All chemicals belonging to the first four groups suppressed the induction of the TRα transcript by T₃ at the indicated concentrations. Therefore, the chemicals in the fourth group would target some process other than the two processes we investigated here. The fifth group contains those chemicals that inhibited T₃ uptake but not T₃ binding to TR and that did not suppress the induction of TRα transcript by T₃. Di-*n*-butyl phthalate, a chemical belonging to the fifth group, activated synergically the induction of the TRα transcript with T₃ by an unknown mechanism. These results indicate that thyroid-disrupting chemicals target several processes within the thyroid system in a chemical-dependent manner, although at present we cannot elucidate which process when inhibited by the chemicals exerts the most profound effect on the thyroid system. The present study raises the possibility that the T₃-uptake system could be a candidate target for these chemicals belonging to the first and second groups.

From the present study, we cannot evaluate the metabolic effect of these chemicals that might occur in organisms. Further studies are necessary for understanding the effects of the chemicals on cells or organisms for an extended period of time. We examined the V_i of saturable [125 I] T_3 uptake for the first 2-min period and the induction of the *tra* gene in 2-day cultured RBCs, in the presence of EDCs. The difference in the cellular responses to EDCs between the 2-min and 2-day treatments needs to be considered.

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