Characterization of uptake and compartmentalization of 3,5,3'-tri-iodothyronine in cultured neonatal rat cardiomyocytes

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Abstract

The uptake of tri-iodothyronine (T_3) in cultured neonatal rat cardiomyocytes was investigated and compared with the uptake of reverse T_3 (rT_3) and thyroxine (T_4). Cellular compartmentalization of T_3 was studied by distinguishing T_3 activity associated with the plasma membrane from that in the cytosol or incorporated in the cell nucleus. T_3 and T_4 uptake displayed similar temperature dependencies which, in magnitude, differed from that of rT_3 uptake. T_3 uptake was Na^+ independent, and sensitive to oligomycin and monodansylcadaverine (42–49% and 25% inhibition of 15-min cellular uptake respectively). Furthermore, T_3 uptake could be inhibited by tryptophan (20%) and tyrosine (12%), while 2-aminobicyclo[2,2,1]heptane-carboxylic acid had no effect. Co-incubation with tryptophan and oligomycin resulted in an additive inhibition of T_3 uptake (77%). We therefore conclude that (i) T_3 uptake is energy dependent, (ii) receptor-mediated endocytosis may be involved and (iii) the aromatic amino acid

transport system T may play a role, while system L is not involved in T_3 transport in cardiomyocytes. Co-incubation with unlabeled iodothyronines showed that 3,3'-di-iodothyronine and T_3 itself were the most effective inhibitors of T_3 uptake (30% and 36% inhibition of 15-min cellular uptake respectively). At 15-min incubation time, 38% of the total cell-associated T_3 was present in the cytosol and nucleus, and 62% remained associated to the plasma membrane. Unidirectional uptake rates did not saturate over a free T_3 concentration range up to $3.9 \mu M$. We have concluded that T_3 uptake in neonatal rat cardiomyocytes occurs by an energy- and temperature-dependent mechanism that may include endocytosis and amino acid transport system T, and is not sensitive to the Na^+ gradient. Elucidation of the molecular basis for the T_3 transporter is the subject of current investigation.

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Introduction

The heart is a major target organ for thyroid hormone action. Thyroid hormone-induced changes in cardiac function are achieved through activation of iodothyronine-sensitive genes (Dillmann 1990, Gloss *et al.* 1999), or through indirect, non-genomic pathways (Craelius *et al.* 1990, Davis & Davis 1993). Genomic tri-iodothyronine (T_3)-mediated effects result from the association of the hormone to nuclear receptors that modify transcription of specific mRNAs, coding for, e.g. the α -isoform of myosin heavy chain or sarcoplasmic reticulum Ca^{2+} -ATPase (Rohrer *et al.* 1991). A prerequisite for binding to the nuclear receptor is transmembrane transport of the hormone and, in the past, a non-protein-mediated diffusion pathway for T_3 transport has been proposed (Lein & Dowben 1961, Pardridge & Mietus 1980). However, a diffusion pathway is unlikely due to the lipophilic nature of thyroid hormones which makes it energetically unfavorable to cross the membrane unassisted, as

evidenced by results from electron spin resonance studies and experiments on artificial lipid bilayers (Lai *et al.* 1985, Chehín *et al.* 1999). Moreover, a transport mechanism would confer a regulatory step in thyroid hormone metabolism (Hennemann *et al.* 1998). For example, in patients with non-thyroidal illness or during starvation, plasma T_3 production by thyroxine (T_4) deiodination in the liver is decreased (Docker & Krenning 1990), and Hennemann *et al.* (1993) showed that this resulted from a reduced cellular uptake of T_4 , not from defective 5'-deiodination. Thus, the transmembrane transport of thyroid hormones is important in the overall body thyroid hormone bioactivity.

Although the heart is one of the most responsive target organs to thyroid hormones, little is known about the mechanisms of transport of these hormones across the plasma membrane of the cardiomyocyte. During the past decade evidence has been presented for T_3 uptake through a pathway mediated by a specific carrier protein (Oppenheimer & Schwartz 1985, Docker *et al.* 1987, Pontecorvi & Robbins 1989, Kragie 1994, Everts *et al.*

1996b) and for receptor-mediated endocytosis (Cheng 1983a). To our knowledge, only our previous report examines T_3 transport in cardiomyocytes (Everts *et al.* 1996b). In the current report, we continue the investigation on the transport of T_3 in the heart and its compartmentalization, using cultured neonatal cardiomyocytes of the rat as a model system. Since, in some organs, T_3 and T_4 share the same transporter (Everts *et al.* 1996a), uptake of T_3 in cardiomyocytes was compared with that of T_4 . The substrate specificity of the putative transporter was further assessed by comparing the uptake of T_3 with that of reverse T_3 (rT_3), and by testing the effects of 3,5-di-iodothyronine (3,5- T_2) and 3,3'-di-iodothyronine (3,3'- T_2). Furthermore, a growing number of studies demonstrate that uptake systems for aromatic amino acids, e.g. system L and system T, are involved in the transport of thyroid hormones (Everts *et al.* 1994, Mitchell *et al.* 1999, Ritchie *et al.* 1999). In our previous report, we investigated the effect of tyrosine and tryptophan on T_3 uptake and suggested that part of the T_3 uptake may occur through these systems (Everts *et al.* 1996b). In the present report, we have continued these studies by examination of the dose-dependency of the effect of tryptophan (Trp), tyrosine (Tyr) and 2-aminobicyclo[2,2,1]heptane-carboxylic acid (BCH), a system L inhibitor, on T_3 uptake.

Materials and Methods

Animals

Wistar rats, 3-days-old and of both sexes, were obtained from laboratory stock (Utrecht University, The Netherlands). They were killed by decapitation, and the hearts were quickly dissected and processed as described below to obtain primary cultures of cardiomyocytes.

Materials

All reagents used for cell isolation and cell culture were obtained from Life Technologies BV (Breda, The Netherlands), with the exception of trypsin and deoxyribonuclease which were purchased from Boehringer (Mannheim, Germany). Polystyrene culture dishes (24 wells) were obtained from Corning Costar Europe (Badhoevedorp, The Netherlands). Iodothyronines (T_4 , T_3 , 3,3'- T_2 , 3,5- T_2), monodansylcadaverine (MDC), oligomycin, ouabain, Trp, Tyr, sulfobromophthalein (BSP) and BCH were purchased from Sigma Chemical Co. (St Louis, MO, USA). [^{125} I] T_3 (81.4 TBq/mmol), [^{125} I] rT_3 (24.4 TBq/mmol) and [^{125} I] T_4 (4.3 TBq/mmol) were purchased from NEN Life Science Products, Inc. (Boston, MA, USA).

Cell culture

Primary cultures of neonatal rat cardiomyocytes were prepared using a trypsin digestion method as described

previously (Everts *et al.* 1996b, Verhoeven *et al.* 2001). An enriched myocardial fraction was obtained by including a preplating step (60 min at 37 °C; 5% CO₂; in 250 ml culture flasks) to remove fibroblasts (Blondel *et al.* 1971). After this procedure, the preparation consisted of more than 90% cardiomyocytes (van Heugten *et al.* 1994). Cardiomyocytes were seeded into 24-well culture dishes at a density of 1.0×10^6 cells per well, and cultured at 37 °C in a 5% CO₂ atmosphere in 4:1 (w/w) Dulbecco's modified Eagle's medium (DMEM)–medium 199 (M199) supplemented with 5% horse serum (HS), 5% fetal calf serum (FCS), and 2% penicillin/streptomycin. One day after isolation the culture medium was replaced by fresh medium. Experiments were routinely performed after 5 days of culture at which time the cardiomyocytes reached confluency and were in a spontaneously and synchronously contracting monolayer (Verhoeven *et al.* 2001). Cell density was virtually constant, as the amount of total cellular protein per well (30–40 µg) varied only slightly between preparations. Cell viability on the day of experimentation, as judged by the ability of cells to exclude trypan blue, was virtually 100%. Although culture in the presence of serum might result in proliferation of fibroblasts that also show active uptake of [^{125} I] T_3 and [^{125} I] T_4 (Docker *et al.* 1987), it has previously been shown that uptake of [^{125} I] T_3 and [^{125} I] T_4 was similar in cardiomyocytes cultured in the absence and presence of serum (Everts *et al.* 1996b). Moreover, the uptake of [^{125} I] T_3 expressed per µg protein is more than tenfold higher in cardiomyocytes than in cardiac fibroblasts (S van der Heide, personal communication).

Cellular uptake studies

After removal of the culture medium, cardiomyocytes were preincubated for 30 min at 37 °C in 0.5 ml Krebs–Ringer buffer (139 mM NaCl, 4.2 mM KCl, 1.5 mM CaCl₂, 1.4 mM KH₂PO₄, 1.4 mM MgSO₄, 5.0 mM glucose; prepared in Tris at pH 7.4) supplemented with 0.5% bovine serum albumin (BSA). Incubation was initiated by quickly replacing the preincubation medium with 0.5 ml Krebs–Ringer buffer containing the additions described below and [^{125} I] T_3 (4×10^5 c.p.m./ml; 82 pM), [^{125} I] rT_3 (4×10^5 c.p.m./ml; 273 pM) or [^{125} I] T_4 (4×10^5 c.p.m./ml; 1.6 nM). The concentrations of BSA and iodothyronines in our incubation media were chosen so as to resemble free plasma hormone concentrations in the neonatal rat. Incubations were performed at 37 °C, and the culture wells were mildly agitated. Uptake was terminated by quick aspiration of the incubation medium, and culture wells were rinsed three times with 1 ml ice-cold saline. Cells were dissolved in 1 ml 0.1 M NaOH and analyzed for ^{125} I activity in a gamma-counter (Packard Cobra II, Packard Instruments Co., Meriden, CT, USA). The time-course of T_3 uptake was assayed in the presence or absence of sodium. Sodium-free conditions were

attained by replacing sodium with choline on a mol-for-mol basis in the preincubation and incubation media. To test the temperature dependency of iodothyronine uptake, cardiomyocytes were preincubated and incubated at 0, 20 or 37 °C. Unidirectional iodothyronine uptake was measured by incubation of cardiomyocytes for 5 min in Krebs–Ringer buffer containing [^{125} I] T_3 and increasing concentrations of the unlabeled hormone (10 nM–100 μ M). Experiments in which the effect of analogues on 15-min T_3 uptake was measured were performed by adding 10 μ M of the analogue to the Krebs–Ringer buffer containing 4×10^5 c.p.m./ml [^{125} I] T_3 . In experiments in which the effects of oligomycin and MDC were investigated, they were added to both the preincubation and incubation medium. Possible involvement of other known transport systems in the uptake of [^{125} I] T_3 was examined by preincubation and incubation with 0.5 or 2 mM Tyr or Trp, 10 or 25 μ M BSP, or 5 mM BCH.

The amount of [^{125} I] T_3 , [^{125} I] rT_3 or [^{125} I] T_4 associated with the cells was expressed as either percentage of added ^{125} I activity (percentage of dose) or as pmol/ 10^6 cells. Results were corrected for the amount of ^{125} I activity associated with the walls of the culture wells by subtracting the amount of ^{125} I activity extracted from the wells incubated without cells.

Compartmentalization studies

To distinguish [^{125} I] T_3 activity partitioned within the plasma membrane from [^{125} I] T_3 activity present in the cytosol, cardiomyocytes were treated with detergent to release cytosolic tracer contents. After incubation in Krebs–Ringer buffer, as described above, cells were treated at room temperature with a permeabilization buffer containing 50 μ g/ml saponin in 160 mM NaCl and 5 mM $MgCl_2$ (pH 7.4). During permeabilization, the incubation wells were mildly agitated to ensure efficient exchange of cellular contents with the overlying medium. Saponin treatment effectively permeabilized the cells, as judged by the failure of treated cells to exclude trypan blue. Total protein content of cardiomyocytes did not change after permeabilization, indicating that saponin did not solubilize cells. After 30 min, the medium was aspirated and analyzed for [^{125} I] T_3 activity, which we interpreted to originate from the cytosol and, hence, resulting from the activity of the putative plasma membrane T_3 transport mechanism. After permeabilization, the remaining cellular debris was dissolved in 1 ml 0.1 M NaOH and analyzed for [^{125} I] T_3 activity. We interpreted the latter activity to be mainly associated to the plasma membrane.

To isolate cell nuclei, the cellular debris remaining after treatment with saponin was scraped from the well using a rubber spatula and collected in 1 ml ice-cold phosphate-buffered saline (PBS), pH 7.4 (42.5 mM Na_2HPO_4 , 7.5 mM NaH_2PO_4 , 150 mM NaCl). The well was rinsed once with 1 ml ice-cold PBS, and both 1 ml fractions were

pooled and centrifuged at 300 *g* for 7 min at 4 °C. The pellet thus obtained was dissolved and thoroughly mixed in 1 ml 0.5% (v/v) Triton X-100 in PBS and centrifuged at 900 *g* for 5 min at 4 °C. This yielded a pellet consisting of nuclei with virtually no contamination by membrane or organelle fragments as judged from protein and DNA measurements. Pellet and supernatant were analyzed separately for [^{125} I] T_3 activity. All results were corrected for the amount of [^{125} I] T_3 activity collected from the wells in the absence of cells.

Protein and DNA measurements

Cellular protein was measured according to the method of Lowry *et al.* (1951). DNA was quantified using a Hoechst fluorescence staining method (Downs & Wilfinger 1983).

Free hormone fraction measurements

Calculations of free T_3 , rT_3 and T_4 concentrations were based on the determinations of the free hormone fraction by equilibrium dialysis (Sterling & Brenner 1966). In Krebs–Ringer buffer containing 0.5% BSA and 82 pM [^{125} I] T_3 , 273 pM [^{125} I] rT_3 or 1.6 nM [^{125} I] T_4 , the free hormone fractions were $2.39 \pm 0.2\%$ ($n=6$), $1.46 \pm 0.02\%$ ($n=3$) and $0.58 \pm 0.04\%$ ($n=3$) respectively (means $\pm$ s.d.). Furthermore, the free [^{125} I] T_3 fractions in the presence of 1, 10 or 100 μ M unlabeled T_3 were $2.54 \pm 0.4\%$, $2.92 \pm 0.5\%$ and $3.90 \pm 0.4\%$ ($n=3$) respectively (means $\pm$ s.d.). From the latter data a calibration curve was constructed to calculate unknown free [^{125} I] T_3 fractions by interpolation.

Statistics and calculations

A single experiment consisted of duplicate or triplicate observations. Data are presented as means $\pm$ s.e.m. unless stated otherwise. Statistical significance was evaluated by repeated measures ANOVA or Student's *t*-test, where appropriate. Statistical significance was accepted at $P < 0.05$. Transport data were analyzed using a non-linear regression data analysis program.

Results

[^{125} I] T_3 uptake and Na^+ dependency

Figure 1 shows the time-course of [^{125} I] T_3 uptake. Hormone uptake leveled off after 30 min. No significant difference was found between uptake of [^{125} I] T_3 in the presence of 139 mM Na^+ or an equimolar concentration of choline in the incubation medium, indicating that T_3 uptake was not Na^+ dependent. This was corroborated by the observation that preincubation and incubation of

Figure 1 Time-course of [¹²⁵I]T₃ uptake in neonatal cardiomyocytes. Cardiomyocytes from 3-day-old rats were cultured at a density of 1×10^6 cells/well for 5 days in DMEM/M199 supplemented with 5% FCS–5% HS. The uptake of [¹²⁵I]T₃ (4×10^5 c.p.m./ml; 82 pM) was measured in Krebs–Ringer buffer with Na⁺ (●) or in Krebs–Ringer buffer in which Na⁺ was replaced by choline (○), both supplemented with 0.5% BSA. Incubations were performed at 37 °C. Means $\pm$ S.E.M. of three experiments are shown.

cardiomyocytes with 1 mM ouabain, an inhibitor of the plasma membrane sodium pump, did not affect the uptake of T₃ (data not shown).

Effects of temperature on iodothyronine uptake

Figure 2 shows the temperature sensitivity of iodothyronine uptake by neonatal cardiomyocytes measured at 5-min incubation time. Cellular [¹²⁵I]T₃ uptake values, expressed as a percentage of the dose, increased 3.6- to 3.8-fold from 0 to 20 °C and 2.2- to 2.8-fold from 20 to 37 °C ($P < 0.001$). Uptake of [¹²⁵I]rT₃ at 20 °C was increased compared with 0 °C; however, uptake did not rise significantly upon increasing the temperature from 20 to 37 °C (Fig. 2A).

Figure 2B shows that uptake of [¹²⁵I]T₄, expressed as a percentage of the dose, rose 2.8- and 2.1-fold upon increases in incubation temperature from 0 to 20 °C and from 20 to 37 °C respectively, the only statistically significant increase being from 0 to 37 °C. Compared with [¹²⁵I]T₄, uptake of [¹²⁵I]T₃ at 37 °C was 2.3-fold higher.

Calculations of free T₃, rT₃ and T₄ concentrations were based on determinations of the free fractions by equilibrium dialysis performed at 37 °C. Uptakes at 37 °C of T₃, T₄ and rT₃ expressed in fmol/pM free hormone were 0.36 ± 0.12 ($n = 7$), 0.70 ± 0.28 ($n = 4$) and 0.21 ± 0.03 ($n = 3$) respectively.

Figure 2 Effect of temperature in neonatal rat cardiomyocytes measured at 5-min incubation time of (A) [¹²⁵I]T₃ (solid bars) and [¹²⁵I]rT₃ (hatched bars) ($n = 3$) and (B) [¹²⁵I]T₃ (solid bars) and [¹²⁵I]T₄ (open bars) ($n = 4$). Uptake values are expressed as a percentage of the dose. † $P < 0.05$ vs 0 °C; * $P < 0.05$ vs 20 °C.

Effects of thyroid hormone analogues

Fifteen-minute uptake of [¹²⁵I]T₃ (total concentration: 82 pM) was significantly inhibited by 10 μ M unlabeled T₃ (36%, $P < 0.05$, $n = 5$) and 10 μ M 3,3'-T₂ (30%, $P < 0.05$, $n = 4$), while the effects of 3,5-T₂ (20%, $n = 4$) and T₄ (22%, $n = 4$) were not significant (data not shown).

Effects of inhibitors on [¹²⁵I]T₃ uptake

To test the energy dependence of [¹²⁵I]T₃ uptake and the possible involvement of endocytosis, cardiomyocytes were

Figure 3 Effects of 10 and 50 µM oligomycin on 5-min ($n=3$), and on 15-min [^{125}I] T_3 uptake ($n=4$). Oligomycin was present during the preincubation (30 min) and incubation. * $P<0.05$ vs control; ** $P<0.01$ vs control.

incubated for 5 or 15 min with either 10 or 50 µM oligomycin, or 100 µM MDC. Oligomycin at a concentration of 50 µM significantly inhibited [^{125}I] T_3 uptake by 44% ($P<0.05$) at 5 min and by 42–49% ($P<0.001$) at 15 min (Fig. 3 and Table 1). MDC, an inhibitor of receptor-mediated endocytosis, reduced the uptake of [^{125}I] T_3 by 25% at 15 min ($P<0.001$) (Table 1). To examine whether oligomycin and MDC affected T_3 transport through separate mechanisms, cardiomyocytes were preincubated and incubated for 15 min with 50 µM oligomycin together with 100 µM MDC. This

Figure 4 Effect of 0.5 and 2 mM Trp, 0.5 and 2 mM Tyr and 5 mM BCH on 15-min uptake of [^{125}I] T_3 . Substrates were present during preincubation and incubation. Data show means $\pm$ S.E.M. of two to four experiments. * $P<0.05$ vs control; ** $P<0.001$ vs control.

co-incubation resulted in a 47% reduction of T_3 uptake, which was comparable to the effect of oligomycin alone (Table 1).

Participation of other transporters in [^{125}I] T_3 uptake; effects of Trp, Tyr, BCH and BSP

To test the possible involvement of amino acid transport systems T and L in the uptake of [^{125}I] T_3 , cardiomyocytes were preincubated and incubated (15 min) with 0.5 or 2 mM Trp, 0.5 or 2 mM Tyr (substrates for system T and L) or 5 mM BCH, a specific system L inhibitor (Broer *et al.* 1998) (Fig. 4). Trp and Tyr showed a dose-dependent inhibition of [^{125}I] T_3 uptake (20% and 12% maximal inhibition), of which the effect of Trp was significant (0.5 mM, $P<0.05$; 2 mM, $P<0.001$). Incubation with BCH showed no reduction of 15-min [^{125}I] T_3 uptake (Fig. 4) nor of 60-min [^{125}I] T_3 uptake (data not

Table 1 Effects of oligomycin, MDC, Trp and combinations of these compounds on 15-min uptake of [^{125}I] T_3 . Data show the means $\pm$ S.E.M. of two to four experiments (shown in parentheses)

Experimental conditions	[^{125}I] T_3 uptake (% dose)	Effect (%)	P
No additions	3.82 $\pm$ 0.05 (4)		
+50 µM oligomycin	1.93 $\pm$ 0.13 (4)	–49	<0.001
+100 µM MDC	2.87 $\pm$ 0.11 (4)	–25	<0.001
+2 mM Trp	2.98 $\pm$ 0.07 (3)	–22	<0.001
+50 µM oligomycin+100 µM MDC	2.04 $\pm$ 0.17 (3)†	–47	<0.001
+50 µM oligomycin+2 mM Trp	0.87 $\pm$ 0.07 (2)*	–77	<0.001

The cardiomyocytes were preincubated (30 min) and incubated (15 min) in the absence or presence of oligomycin, MDC, Trp, oligomycin with MDC or oligomycin with Trp. The inhibitory effect of these compounds on [^{125}I] T_3 uptake is compared with no additions and expressed as %. Statistical differences between additions and no additions are indicated. † $P<0.001$, oligomycin+MDC vs MDC alone; * $P<0.001$ oligomycin+Trp vs oligomycin or Trp alone.

shown). The combined effect of 50 μM oligomycin and 2 mM Trp was tested in two experiments and showed a larger and additive reduction of $[^{125}\text{I}]\text{T}_3$ uptake (77%, $P < 0.001$) compared with the effects of the two compounds alone (49% and 22% respectively) (Table 1). In addition, possible participation of an organic anion transport system in the uptake of $[^{125}\text{I}]\text{T}_3$ was examined in two independent experiments by preincubation and incubation for 5 min with 10 or 25 μM BSP. BSP is transported by members of the family of organic anion transporters (OATP family) (Wolkoff 1996). This gave the following values as percentage of the dose: controls, 2.36 ± 0.16 ($n=8$); 10 μM BSP, 2.51 ± 0.18 ($n=4$); 25 μM BSP, 2.22 ± 0.30 ($n=4$), showing that BSP does not inhibit T_3 uptake.

Substrate-dependent unidirectional uptake

From Fig. 1 we chose a time-point of 5 min to measure unidirectional T_3 uptake rates. Figure 5A shows that unidirectional uptake rate of T_3 , although not adequately described by a straight line, does not saturate over a total T_3 concentration ranging from 10 nM to 100 μM . When T_3 uptake data were analyzed as a function of the free T_3 concentration, the curve describing cellular uptake deviated notably from a straight line (Fig. 5B). Although uptake data appeared to converge on a rectangular hyperbola, the curve could not be adequately described by simple saturation kinetics.

Compartmentalization of T_3

Our observation of the apparent non-saturability of cellular T_3 uptake rates prompted us to investigate the passive incorporation of the lipophilic tri-iodothyronine in the plasma membrane as a possible confounding factor in our uptake assays. After an incubation period of 15 min and following detergent treatment of the cardiomyocytes, 35% of the total $[^{125}\text{I}]\text{T}_3$ activity in the cells was retrieved in the cytosolic fraction. The plasma membrane fraction contained 62% of the total $[^{125}\text{I}]\text{T}_3$ activity, and 3% was incorporated in the nuclei. After an incubation period of 120 min, total cellular $[^{125}\text{I}]\text{T}_3$ activity was increased 2.2-fold compared with 15 min. Now, 50% of the total $[^{125}\text{I}]\text{T}_3$ activity was associated with the membrane, and 47% was recovered from the cytosolic fraction, while 3% was incorporated into the nuclei (Table 2). In one experiment we determined the recovery of DNA in the nuclear fraction. As the DNA content of intact cells amounted to $6.8 \pm 0.6 \mu\text{g DNA}/0.5 \times 10^6$ cells ($n=3$), DNA content of the isolated nuclear fractions after 15 and 120 min amounted to $4.9 \pm 0.3 \mu\text{g DNA}/0.5 \times 10^6$ cells ($n=3$) (72% recovery) and to $5.0 \pm 0.1 \mu\text{g DNA}/0.5 \times 10^6$ cells ($n=3$) (74% recovery) respectively.

We again determined the unidirectional T_3 uptake rate as a function of the free T_3 concentration, but now

Figure 5 Unidirectional T_3 uptake rates as a function of (A) the total T_3 concentration and (B) the calculated free T_3 concentration. The dotted line shows the result of a non-linear regression analysis of the data points to the linear function $y=ax+b$, indicating the deviation of the data from a straight line. Data points represent the mean $\pm$ S.E.M. of three to seven experiments. Please note the different abscissa ranges in panels A and B.

measuring the $[^{125}\text{I}]\text{T}_3$ activity released from the cytosol by detergent treatment and the $[^{125}\text{I}]\text{T}_3$ activity remaining in the plasma membrane after permeabilization. Figure 6 shows that the uptake rates measured in the membrane-bound and cytosolic fraction did not saturate over a free T_3 concentration ranging from 25 nM to 3.9 μM .

Discussion

The main outcome of this study was that the uptake of T_3 in neonatal rat cardiomyocytes (cultured with serum) is temperature dependent, MDC, oligomycin and Trp sensitive, but not dependent on the Na^+ gradient. Apart from the Na^+ dependency (see below), this confirms our observations in cardiomyocytes cultured in the absence of serum

Table 2 Compartmentalization of T₃ in membrane, cytosol and nucleus of cardiomyocytes. Cardiomyocytes were incubated and treated with saponin as described in Materials and Methods. Data show the mean ± s.d. of two (15-min uptake) to four (120-min uptake) different preparations

Fraction	[¹²⁵ I]T ₃ uptake (% dose)	% of whole cellular uptake
15 min		
Membrane	1.4 ± 0.03	62 ± 0.1
Cytosol	0.8 ± 0.02	35 ± 0.1
Nucleus	0.06 ± 0.001	3 ± 0.1
Total	2.2 ± 0.06	100
120 min		
Membrane	2.5 ± 0.5	50 ± 6
Cytosol	2.3 ± 0.2	47 ± 6
Nucleus	0.17 ± 0.03	3 ± 0.4
Total	5.0 ± 0.43	100

Figure 6 Unidirectional T₃ uptake rates in the membrane-bound (○) and cytosolic (●) fractions of cultured neonatal cardiomyocytes as a function of the free T₃ concentration. Data points represent the means±s.e.m. of three experiments.

(Everts *et al.* 1996*b*). The present study also included examination of the cellular compartmentalization of T₃ showing that, although a high amount of T₃ is associated with the plasma membrane, T₃ is transported into the cytosol and to the nucleus. Finally, T₃ uptake did not saturate at very high free hormone concentrations.

In our first study on thyroid hormone uptake in cardiomyocytes (Everts *et al.* 1996*b*), T₃ uptake was found partly to be dependent on the Na⁺ gradient as judged from the inhibitory effects (20–30%) of 10 μM monensin. In the present study where Na⁺ dependency was judged from incubations with choline buffer, no such Na⁺ dependency could be demonstrated. A similar discrepancy was previously observed when [¹²⁵I]T₄ uptake by cultured anterior pituitary cells was examined (Everts *et al.* 1994).

While [¹²⁵I]T₄ uptake was reduced by 40% in the presence of 10 μM monensin, it was reduced by only 20% in choline-containing buffer (Everts *et al.* 1994). Probably, by inducing the Na⁺ influx, monensin causes a more pronounced rundown of the Na⁺ gradient across the plasma membrane, as compared with replacing the Na⁺ by choline.

The present results indicate that neonatal rat cardiomyocytes possess a specific T₃ uptake system, as evidenced by the fact that T₃ itself is the most potent inhibitor of [¹²⁵I]T₃ uptake and by the difference in temperature sensitivities between the uptake of T₃ and its analogue rT₃. A plasma membrane receptor or an integral membrane carrier protein would confer substrate specificity to the T₃ transport mechanism but, to date, no such protein has been identified. It has been found, however, that a monoclonal antibody directed against an M_r 52 000 membrane protein inhibited T₃ and T₄ uptake in rat liver cells (Mol *et al.* 1986). This coincides remarkably with the observation that two M_r 53 000 and 55 000 membrane-associated proteins were sensitive to N-bromoacetyl-[¹²⁵I]T₃ affinity labeling in cultured mouse fibroblasts (Cheng 1983*b*), a cell type shown to take up T₃ by receptor-mediated endocytosis (Cheng 1983*a*). It remains to be investigated whether these observations are the manifestations of the putative T₃ transporter/receptor.

In whole cell uptake experiments, a large amount of T₃ activity was associated to the plasma membrane. Similar findings were reported by others (Yusta *et al.* 1988, Chehín *et al.* 1999), and this is in line with the known lipophilicity of native iodothyronines (Pontecorvi & Robbins 1989). The presence of this (non)-specific binding of substrate to the plasma membrane could explain why the time-course of T₃ uptake could not be adequately described by a single exponential. These results would imply that passive partitioning of the substrate in the lipid bilayer of the plasma membrane masks the uptake performed by a specific transport mechanism, thus confounding the results of whole cell uptake studies. In addition, several groups have described the presence of receptors at the plasma membrane which initiate non-genomic effects, e.g. modulation of Na⁺ channels (Craelius *et al.* 1990, Davis & Davis 1993). Association of T₃ with these receptors presumably excludes uptake of the hormone. This warrants the use of a method which distinguishes membrane associated (non)-specific activity from that translocated across the plasma membrane by a transport system. We used a detergent treatment to determine this non-specific activity, resulting in values comparable with those reported by others (Cheng 1983*a*, Pontecorvi & Robbins 1989). It is noteworthy that the magnitude of the unlabeled T₃-inhibitable component in whole cell uptake (i.e. 36%, see Results) is comparable with the fraction of T₃ activity released from cardiomyocytes by detergent treatment (i.e. 35%, see Table 2), both measured at 15 min. Measurements after 2 h of incubation showed

similar values (37%) for the reduction of whole cell uptake by unlabeled T_3 (Verhoeven *et al.* 2001). However, the large effect (80%) on nuclear T_3 binding after this incubation period (Verhoeven *et al.* 2001) and the increase of T_3 activity in the cytosol (this report) indicate that T_3 uptake is regulated by a specific mechanism at the plasma membrane.

Data on plasma thyroid hormone, especially rT_3 , concentrations in the neonatal rat are scant. From data in the literature we estimated values of 2–7 pM free T_3 , 2–17 pM free T_4 and 1 pM free rT_3 respectively (Rutgers *et al.* 1987, Rondeel *et al.* 1988, Aláez *et al.* 1992, Schröder-van der Elst & van der Heide 1992), and we chose the composition of our incubation media to approximate to the free plasma hormone concentrations in the neonatal rat. The cardiomyocytes were exposed to 2 pM free T_3 , 10 pM free T_4 or 4 pM rT_3 . This allowed us to compare thyroid hormone uptake in cardiomyocytes under physiological conditions. T_3 uptake at 37 °C, expressed as percentage of dose, was higher than the uptake of T_4 and rT_3 (around two- and fourfold respectively). These results indicate that, under physiological conditions *in vitro*, cardiomyocytes take up thyroid hormone with a distinct preference for T_3 over T_4 and rT_3 . Taking into account the differences in experimental conditions (e.g. different BSA and total hormone concentrations, seeded cell densities), the present uptake values for T_4 and T_3 corresponded well with those reported in our previous study (Everts *et al.* 1996b).

The inhibition of T_3 uptake by MDC hints at the involvement of receptor-mediated endocytosis in the transmembrane transport of the hormone in cardiomyocytes. This is corroborated by results from other studies presenting evidence for a physiological role for endocytosis in the uptake of substrates in rat cardiomyocytes (Iida & Shibata 1989, Page *et al.* 1994). Not only in our cardiomyocyte preparation but also in other cell types, MDC was found to inhibit the uptake of T_3 (Goncalves *et al.* 1989, Pontecorvi & Robbins 1989, Everts *et al.* 1993, McLeese & Eales 1996), suggesting that receptor-mediated endocytosis participates in cellular T_3 uptake. The inhibitory effects of MDC and oligomycin on T_3 uptake were not additive. Oligomycin affects the energy status of the cardiomyocyte by reducing its ATP content. Receptor-mediated endocytosis is an energy-dependent process (Barouch *et al.* 1994), and this would explain the inhibition by oligomycin. Further examination of the involvement of this process is the subject of current studies.

Neither in the presence nor in the absence of Na^+ could the uptake of T_3 be adequately described by a single exponential, indicating the involvement of more than one component in T_3 uptake by cardiomyocytes. Interestingly, the effects of co-incubation with oligomycin and Trp on T_3 uptake were additive. It has been shown previously that members of the organic anion transporter family (rat

Oatp1–3, human OATP-C or liver-specific organic anion transporter (LST-1)) transport thyroid hormones in a Na^+ -independent manner (Abe *et al.* 1996, Friesema *et al.* 1999). Expression profiles show that some members of the OATP family are localized in the heart (Tamai *et al.* 2000). Furthermore, Abe *et al.* (1996) showed that BSP, which is an organic anion, inhibits OATP-mediated uptake of thyroid hormones. T_3 uptake in neonatal cardiomyocytes was not affected by BSP, indicating that this type of transporter is not involved in uptake of thyroid hormones in rat heart. Other likely candidates for a putative accessory transport system could be members of amino acid transport systems T or L. Both system T and L are Na^+ independent, and both systems transport aromatic amino acids (Kragie 1994, Palacín *et al.* 1998). It has been shown that the aromatic amino acids Tyr and Trp, which can be transported by both systems L and T, inhibit the uptake of T_3 in neonatal cardiomyocytes and transfected *Xenopus* oocytes (Everts *et al.* 1996b, Ritchie *et al.* 1999). Only system L is sensitive to the synthetic amino acid analogue BCH (Broer *et al.* 1998, Ritchie *et al.* 1999). Our results show that uptake of T_3 was not sensitive to BCH, from which we conclude that system L does not participate in uptake of T_3 in neonatal rat cardiomyocytes. However, based on the inhibition of T_3 uptake by Trp and Tyr shown in this report and the previous study in neonatal rat cardiomyocytes (Everts *et al.* 1996b), we cannot exclude system T as a candidate for a putative accessory transport system. We therefore plan to explore the participation of system T in uptake of thyroid hormones in cardiomyocytes further.

In summary, the results of our study on neonatal cardiomyocytes indicate the presence of an energy- and temperature-dependent uptake mechanism for T_3 that is not dependent on the transmembrane Na^+ gradient. Based on the inhibitory effect of MDC, an endocytosis-mediated uptake mechanism cannot be excluded. Our data also suggest the presence an additional uptake system, e.g. amino acid transport system T. We are currently deploying an expression-cloning strategy to further characterize the T_3 uptake mechanism in neonatal rat cardiomyocytes.

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