Uptake of triiodothyronine and triiodothyroacetic acid in neonatal rat cardiomyocytes: effects of metabolites and analogs

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Abstract

Cellular and nuclear uptake of [^{125}I]tri-iodothyronine (T_3) and [^{125}I]triiodothyroacetic acid (Triac) were compared in cardiomyocytes of 2–3 day old rats, and the effect of thyroid hormone analogs on cellular T_3 uptake was measured. Cells ($5–10 \times 10^5$ per well) were cultured in DMEM–M199 with 5% horse serum and 5% FCS. Incubations were performed for from 15 min to 24 h at 37 °C in the same medium, 0·5% BSA and [^{125}I]T_3 (100 pM), or [^{125}I]Triac (240 pM). Expressed as % dose, T_3 uptake was five times Triac uptake, but expressed as fmol/pM free hormone, Triac uptake was at least 30% ($P<0·001$) greater than T_3 uptake, whereas the relative nuclear binding of the two tracers was comparable. The 15 min uptake of [^{125}I]T_3 was competitively inhibited by 10 μM unlabeled T_3 (45–52%; $P<0·001$) or 3,3′-diiodothyronine (T_2) (52%; $P<0·001$), and to a smaller extent by thyroxine (T_4) (27%; $0·05<P<0·1$). In contrast, 10 μM 3,5-T_2, Triac, or tetraiodothyroacetic acid (Tetrac) did not affect T_3 uptake after 15 min or after 24 h. Diiodothyropropionic acid (DITPA) (10 μM) reduced 15-min T_3 uptake by about 24% ($P<0·05$), but it had a greater effect after 4 h (56%; $P<0·001$). Exposure to 10 nM DITPA during culture reduced cellular T_3 uptake, as did 10 nM T_3, suggesting down-regulation of the plasma membrane T_3 transporters. We conclude that i) Triac is taken up by cardiomyocytes; ii) 3,3′-T_2 and, to a lesser extent, DITPA and T_4 interfere with plasma membrane transport of T_3, whereas 3,5-T_2, Triac, or Tetrac do not; iii) the transport mechanism for Triac is probably different from that for T_3.

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Introduction

Thyroid hormone induces an increase in cardiac contractility and frequency, resulting in a greater cardiac output (Polikar *et al.* 1993, Toft & Boon 2000, Klein & Ojamaa 2001), and a proportional change in energy turnover (Clausen *et al.* 1991). Thyroid hormone also induces relaxation of vascular smooth muscle (Ojamaa *et al.* 1996), and it is very effective in reducing serum cholesterol (Staels *et al.* 1990). Recently, the concept has been discussed that initiation of thyroxine (T_4) replacement therapy may precipitate angina pectoris (Gammage & Franklyn 1997), whereas increased serum triiodothyronine (T_3) at the time of admission to hospital is associated with a greater risk for the development of myocardial ischemia (Peters *et al.* 2000). This underscores the importance of developing thyromimetic compounds with lipid-decreasing activity, without cardiovascular and thermogenic effects (Boyd & Oliver 1960, Underwood *et al.* 1986, Stephan *et al.* 1996, Ichikawa *et al.* 2000, Trost *et al.* 2000).

Patients undergoing coronary bypass surgery (Holland *et al.* 1991, Klemperer *et al.* 1995) and patients with congestive heart failure (Hamilton 1993, Hamilton & Stevenson 1996) may show typical changes in serum thyroid hormone parameters – low serum T_3, high serum reverse T_3 and normal T_4 and thyrotropin (TSH) concentrations – known as the euthyroid sick syndrome (Docter *et al.* 1993). Whether or not this condition should be treated remains a matter of debate (Klemperer *et al.* 1995, Utiger 1995, Camacho & Dwarkanathan 1999). However, during the past 5–10 years, clinical studies have explored the possibility of improving heart function using treatment with T_3 or T_4 (Moruzzi *et al.* 1996, Chowdhury *et al.* 1999, Mullis-Jansson *et al.* 1999), both in children with congenital heart disease and in elderly patients with congestive heart failure. To date, the thyroid hormone analog, diiodothyropropionic acid (DITPA), which possibly has a greater effect on cardiac contractility than on frequency, has been applied in animal studies (Pennock *et al.* 1993, 2000, Morkin *et al.* 1996, Spooner *et al.* 1999).

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With the purpose of suppressing thyrotropin (TSH) secretion in patients with thyroid cancer (Pujol *et al.* 2000), triiodothyroacetic acid (Triac) has been tested for specificity at the pituitary and the peripheral level (Beck-Peccoz *et al.* 1988, Sherman & Ladenson 1992, Everts *et al.* 1994, Sherman *et al.* 1997). In addition, tetraiodothyroacetic acid (Tetrac) was more potent than T_4 in reducing TSH release in isolated pituitary cells (Everts *et al.* 1995) and in hypothyroid rats (Lameloise *et al.* 2001), but it was less efficient in inducing cardiac hypertrophy (Lameloise *et al.* 2001). Recent work on mitochondria indicates that T_3 metabolites such as 3,5-diiodothyronine (3,5-T_2) may have thermogenic effects (Goglia *et al.* 1999). Finally, several studies have demonstrated acute effects of T_3 (Davis & Davis 1993) and 3,5-T_2 (Huang *et al.* 1999) on the heart, in particular on ion channels.

In view of the significance of plasma membrane transport for the bioavailability of thyroid hormones (Hennemann *et al.* 1998, 2001), and the possible existence of different transport systems in different tissues (Kragie 1994, Everts *et al.* 1996*a*), we have previously explored the thyroid hormone transport system in the heart (Everts *et al.* 1996*b*, Van der Putten *et al.* 2001, Verhoeven *et al.* 2001). In the present study we continued this work by comparing the uptake of $[^{125}I]T_3$ with that of $[^{125}I]$Triac in neonatal rat cardiomyocytes. Furthermore, we tested the effects of T_4, the analog DITPA, and the metabolites 3,3′-T_2, 3,5-T_2, Triac and Tetrac on the uptake of $[^{125}I]T_3$.

Materials and Methods

Animals

All experiments were performed using 2–3-day-old Wistar rats of both sexes, obtained from laboratory stock. Rats were killed by decapitation, and hearts were quickly dissected and processed as described below, to isolate cardiomyocytes.

Cell culture

Primary cultures of neonatal rat cardiomyocytes were prepared as described in detail previously (Blondel *et al.* 1971), with some modifications (Van Heugten *et al.*1994, Everts *et al.* 1996*b*, Verhoeven *et al.* 2001). In brief, hearts were cut for 1 min and dissociated with 0·1% trypsin for 10 min at 33 °C. Cells from the first treatment with trypsin were decanted and discarded, and the remaining tissue was further digested with fresh enzyme and decanted. DMEM with medium 199 (M199) (4:1), 5% FCS, 5% horse serum and 2% penicillin/streptomycin was then added to the suspended cells. This procedure was repeated seven times until all tissue was dissociated. Deoxyribonuclease (20 U/ml) was added, and the remaining tissue from the last step, together with the trypsinized cells, was centrifuged (100 ***g***) for 5 min. The supernatant was discarded, and the cells were resuspended in 30 ml DMEM–M199 with 5% FCS, 5% horse serum, and 2% penicillin/streptomycin. The cell suspension was passed through nylon mesh, and the dispersed cells were preplated (Blondel *et al.* 1971) into 250 ml culture flasks for 60 min (37 °C, 5% CO_2) to remove fibroblasts. After this procedure, the preparation consists of more than 90% cardiomyocytes (Van Heugten *et al.* 1994). Cells of the enriched cardiomyocyte fraction were plated into 48-well culture dishes at subconfluent density (5×10^5 cells/well) in 1-ml volumes of DMEM–M199 with 5% FCS–5% horse serum–2% penicillin/streptomycin, and in some cases at a density of 10^6 cells/well in 2-ml volumes in 24-well culture dishes.

The cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C for 1 day. After 1 day, the cells revealed spontaneous and synchronous beating, and the medium was replaced by the original culture medium. Experiments were routinely performed after 5 days. In a series of experiments, the cultured cells were exposed to 10 nM T_3 or DITPA from the start of culture.

Although culturing cardiomyocytes in the presence of serum may result in proliferation of contaminating fibroblasts that also show thyroid hormone transport (Docter *et al.* 1987), we have previously shown that uptake of thyroid hormones is comparable in cardiomyocytes cultured for 5 days in absence or presence of serum (Everts *et al.* 1996*b*, Verhoeven *et al.* 2001). Moreover, the uptake of $[^{125}I]T_3$, expressed per μg protein, is more than 10-fold greater in cardiomyocytes than in cardiac fibroblasts (S M van der Heide, personal communication).

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

The incubation medium was identical to the culture medium, except that serum was replaced by 0·5% BSA for measurements of $[^{125}I]T_3$ or $[^{125}I]$Triac uptake (Everts *et al.* 1994, 1996*b*). Cells were preincubated (0·5 ml) for 30 min and incubated (0·25 ml) for from 15 min to 24 h at 37 °C without or with 10 μM unlabeled T_3, Triac, Tetrac, 3,3′-T_2, 3,5-T_2, or 1 nM–10 μM DITPA, T_3, T_4 or Triac, and in all cases $[^{125}I]T_3$ (100 000 c.p.m.; 100 pM) or $[^{125}I]$Triac (200 000 c.p.m.; 240 pM).

After incubation, the medium was removed and the cells were washed with 1 ml ice-cold saline to remove tracer not bound to the cells. This washing procedure proved to be sufficient (Verhoeven *et al.* 2001). Cells were dissolved in 1 ml 0·1 M NaOH and counted for iodine-125 activity in a 16-channel gamma-counter (NE 1600, Nuclear Enterprises, Edinburgh, UK). The amount of $[^{125}I]T_3$ or $[^{125}I]$Triac 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 recovered from the wells without cells.

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

Cells were cultured at a density of 10^6 cells/well in 24-well culture dishes as described above. Preincubation (30 min) and incubation (2 h) were performed at 37 °C in a volume of 0·5 ml, and [^{125}I]T_3 (200 000 c.p.m.; 100 pM) or [^{125}I]Triac (500 000 c.p.m.; 300 pM) during incubation. Unlabeled T_3 or Triac (both 10 μM) were present only during incubation. After incubation, cells were washed once with 2 ml ice-cold saline. The cells were scraped from the wells with a rubber policeman in 1 ml PBS (on ice) and the wells were washed with 0·5 ml PBS. These two aliquots were combined and centrifuged (300 ***g*** at 4 °C for 7 min) and the cell pellet counted (30 s) and solubilized in 1 ml PBS containing 0·5% Triton X-100 (Everts *et al.* 1996*b*, Verhoeven *et al.* 2001). After 2 min of continuous vortexing, nuclei were spun down (900 ***g*** at 4 °C for 5 min) and washed once with 1 ml PBS containing 0·5% Triton X-100. The nuclear pellets were counted for 5 min and frozen for determination of DNA.

Free hormone concentrations

Calculation of the free hormone concentration was based on determination of the free fraction by equilibrium dialysis (Sterling & Brenner 1966). As shown previously (Everts *et al.* 1994), the free fractions of T_3 and Triac in medium with 0·5% BSA were 3·45 ± 0·19% (n=8) and 0·41 ± 0·03% (n=5) respectively – a ninefold difference. In the present study, the free T_3 fraction was not changed by the addition of 10 μM unlabeled T_3, 3,3′-T_2, 3,5-T_2, Triac or Tetrac, and only slightly by 10 μM T_4 (4·21%; n=2). Upon addition of 0, 1 nM, 10 nM, 100 nM, 1 μM or 10 μM DITPA, the free T_3 fraction also showed a slight increase: from 3·22 ± 0·08% (n=5) to 3·61% (n=2), 3·49% (n=2), 3·45% (n=2), 3·78% (n=2) and 4·36 ± 0·06% (n=3) respectively. The free Triac fraction was 0·38 ± 0·02% (n=6), and did not change with 10 μM unlabeled Triac.

DNA and protein determinations

DNA content was determined using a fluorimetric method (Downs & Wilfinger 1983). The cellular protein content was determined with the Bio-Rad Protein Assay-Kit (Bio-Rad, Munich, Germany). Cells plated at a density of 5×10^5 cells/well contained about 0·1 mg protein/well after 5 days of culture.

Chemicals and isotopes

All reagents used for cell isolation and cell culture were obtained from Gibco Europe (Breda, The Netherlands), with the exception of trypsin and deoxyribonuclease (Boehringer, Mannheim, Germany). Culture dishes (48 and 24 wells) were obtained from Costar (Cambridge, MA, USA). All iodothyronines, 3,5-diiodothyroacetic acid (3,5-Diac), Triac, and Tetrac were obtained from Henning Berlin (Berlin, Germany). BSA (fraction V) and DITPA were purchased from Sigma Chemical Co. (St Louis, MO, USA). [3′-^{125}I]T_3 (3070 μCi/μg) was purchased from Amersham International (Aylesbury, Bucks, UK). [3′-^{125}I]Triac (2730 μCi/μg) was prepared from Na^{125}I and 3,5-Diac using the chloramine-T method (Rutgers *et al.* 1989). Sephadex LH-20 was from Pharmacia (Uppsala, Sweden). All other reagents were of the highest purity available.

Calculations and statistics

On the basis of the free hormone fractions in the incubation buffer with 0·5% BSA, the chemical concentration of T_3 (100 pM) or Triac (240 pM) in the buffer, and the incubation volume, the counts per minute (percentage added dose) were converted to fmol/pM free hormone. In case the free hormone fraction was unknown – for example in the cellular pellet and the nuclei (Table 1) – data were only expressed as percentage of the added radioactivity.

The statistical significance of any of the tested compounds on [^{125}I]T_3 or [^{125}I]Triac uptake was evaluated by Student's t-test or by one-way analysis of variance and Bonferroni's test for multiple comparisons. P<0·05 was regarded as statistically significant.

Results

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

Time-course of cellular uptake Figure 1 shows the time-course of [^{125}I]T_3 uptake (○) compared with that of [^{125}I]Triac (●) in cardiomyocytes, expressed as percentage of the dose (Fig. 1A) and as fmol/pM free hormone (Fig. 1B). Both uptakes showed a steep phase up to 1 h of incubation. Between 1 and 4 h of incubation, [^{125}I]Triac uptake reached equilibrium, whereas [^{125}I]T_3 uptake showed a further increase. As can be seen in Fig. 1A, [^{125}I]T_3 uptake was fivefold greater than that of [^{125}I]Triac, when expressed as percentage of the dose. However, when expressed per pM free hormone (Fig. 1B), the uptake of [^{125}I]Triac was 30% greater than that of [^{125}I]T_3.The free fractions of the two hormones in buffer with 0·5% BSA were 3·5% for [^{125}I]T_3 and 0·4% for [^{125}I]Triac – a ninefold difference.

Nuclear binding To assess nuclear binding of T_3 and Triac, cardiomyocytes were preincubated for 30 min, and incubated for 2 h with [^{125}I]T_3 or [^{125}I]Triac in the absence or presence of 10 μM unlabeled T_3 or Triac (Table 1). Again, cellular uptake expressed as percentage of the dose of [^{125}I]T_3 was fivefold greater than that of

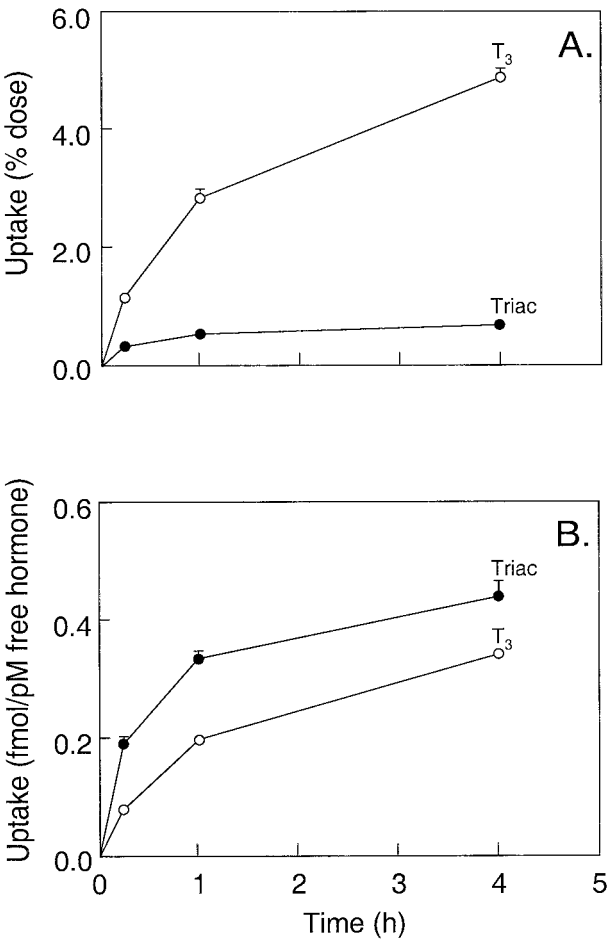

Figure 1 Time-course of uptake of [^{125}I]T_3 (○) and [^{125}I]Triac (●) in neonatal rat cardiomyocytes expressed (A) as % dose and (B) as fmol/pM free hormone. Cardiomyocytes were cultured for 5 days at a density of 500 000 cells/well. Thereafter, culture medium was removed and cells were preincubated for 30 min in incubation medium with 0·5% BSA, followed by incubation in the same medium with [^{125}I]T_3 (100 000 c.p.m./well) or [^{125}I]Triac (200 000 c.p.m./well) for periods of 15 min–4 h. Data represent mean ± S.E. of six observations from two independent experiments.

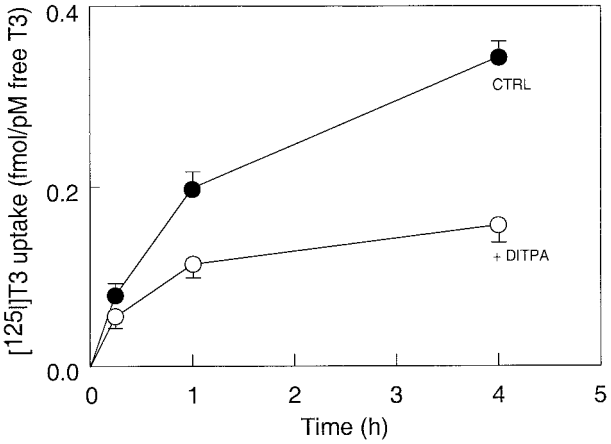

Figure 2 Time-course of the effect of 10 μM DITPA on the uptake of [^{125}I]T_3 in cardiomyocytes. Cells were cultured, preincubated and incubated as described in the legend to Fig. 1, with 10 μM DITPA present during incubation. Data are expressed as fmol/pM free T_3, and represent mean ± S.E. of six observations from two independent experiments. CTRL, control.

[^{125}I]Triac. Incubation with 10 μM unlabeled T_3 or Triac inhibited cellular binding of [^{125}I]T_3 and [^{125}I]Triac, but this effect was smaller than the inhibition of nuclear binding: 19% compared with 72% for T_3, and 34% compared with 60% for Triac. The DNA contents of the nuclear pellets in the absence or presence of 10 μM of the unlabeled hormone were the same.

Effect of DITPA T_3 uptake was also measured over a 4-h incubation period in the absence or presence of 10 μM DITPA (Fig. 2). Results are expressed as fmol/pM free T_3. At incubation times 15 min, 1 h and 4 h, the presence of 10 μM DITPA reduced [^{125}I]T_3 uptake by 28%, 45% and 56% (all $n=6$; $P<0·05$) respectively.

Plasma membrane transport of [^{125}]T_3

Effects of T_3, T_4, Triac, DITPA, Tetrac 3,3′-T_2, and 3,5-T_2 Figure 3 shows the competitive effects of increasing concentrations of unlabeled T_3, T_4, Triac and

Table 1 Cellular and nuclear uptake of [^{125}I] T_3 and [^{125}I]Triac in cultured rat cardiomyocytes

	[^{125}I]T_3 or [^{125}I]Triac activity (% dose)			
	Cell pellet (A)	Nuclear pellet (B)	**(B/A) × 100%**	**DNA content** (ng)
Experimental conditions				
[^{125}I]T_3 (5)	2·20 ± 0·25	0·18 ± 0·04	10·2 ± 2·4	904 ± 118
+10 μM T_3 (6)	1·78 ± 0·13	0·05 ± 0·01	3·1 ± 0·9	1122 ± 110
[^{125}I]Triac (6)	0·41 ± 0·03	0·05 ± 0·01	11·1 ± 3·0	1394 ± 163
+10 μM Triac (6)	0·27 ± 0·01	0·02 ± 0·00	5·6 ± 0·9	1570 ± 182

Data show mean ± S.E. of triplicate observations from two experiments. Cardiomyocytes were prepared from 2-day-old rats, and cultured for 1 day in 24-well dishes in DMEM–M199–5% FCS–5% horse serum at a density of about 10^6 cells/well. After 1 day, the culture medium was changed and culture was continued for 4 days. Cells were incubated at 37 °C for 2 h with [^{125}I]T_3 (200 000 c.p.m.) or [^{125}I]Triac (500 000 c.p.m.) in medium with 0·5% BSA. Cells were scraped from the wells in PBS, centrifuged and the cell pellet (A) counted. The nuclear pellet (B) was obtained after treatment with Triton X-100. The third column shows nuclear [^{125}I]T_3 or [^{125}I]Triac uptake relative to cellular [^{125}I]T_3 or [^{125}I]Triac uptake. The fourth column represents DNA content of the nuclear pellet.

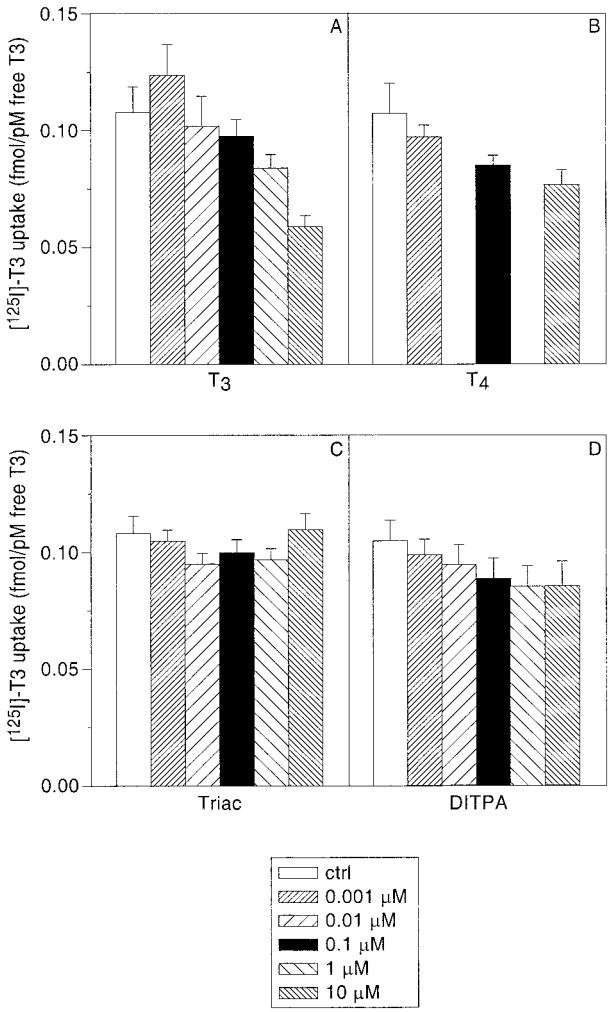

Figure 3 Competitive effects of unlabeled T_3, T_4, Triac and DITPA on the 15-min uptake of [^{125}I]T_3 in cardiomyocytes. The cells were cultured, preincubated and incubated with [^{125}I]T_3 as described in the legend to Fig. 1, with increasing concentrations (1 nM–10 μM) of the unlabeled compounds present during incubation. Data are expressed as fmol/pM free T_3. Bars represent mean ± S.E. of nine observations from three independent experiments. ctrl, control.

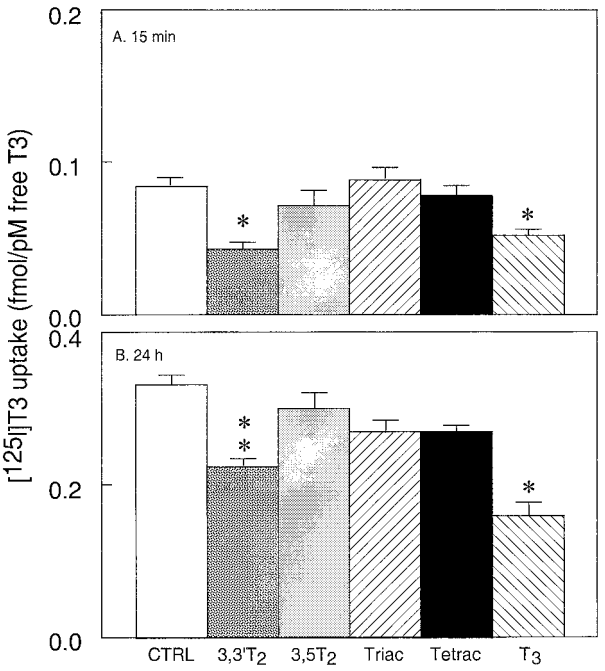

Figure 4 Effects of thyroid hormone metabolites on [^{125}I]T_3 uptake in cardiomyocytes at 15 min (A) and 24 h (B) of incubation. The cells were cultured, preincubated and incubated with [^{125}I]T_3 as described in the legend to Fig. 1, with 10 μM of the unlabeled metabolites present during incubation. Data are expressed as fmol/pM free T_3. Bars represent mean ± S.E. of nine observations from three independent experiments. Significant differences: *P<0·001, **P<0·02 compared with controls (CTRL).

DITPA on the 15-min uptake of [^{125}I]T_3, expressed as fmol/pM free T_3. T_3 itself resulted in a clear dose-dependent inhibition, and the maximum effect was seen with 10 μM (45%; P<0·01) (Fig. 3A). T_4 showed a dose-dependent inhibition, but the maximum effect at 10 μM (27%) was not significant (Fig. 3B). Exposure to Triac did not result in reduction of [^{125}I]T_3 uptake (Fig. 3C), whereas DITPA showed a stepwise reduction with increasing concentrations (Fig. 3D), but the maximum effect was now 20% (NS), as compared with 28% in Fig. 2.

The effects of a number of metabolites tested at a concentration of 10 μM on [^{125}I]T_3 uptake at 15 min (Fig. 4A) and 24 h (Fig. 4B) of incubation are shown in Fig. 4. The uptake curve for [^{125}I]T_3 increased up to 4 h of incubation and reached equilibrium between 4 and 24 h. Under control conditions, [^{125}I]T_3 uptake at 15 min, 1 h, 4 h and 24 h was 0·089 ± 0·017 (n=9), 0·222 ± 0·017 (n=9), 0·433 ± 0·038 (n=9) and 0·352 ± 0·038 (n=9) fmol/pM free T_3 respectively. After 15 min, [^{125}I]T_3 uptake was inhibited by 52% (n=9; P<0·001) in the presence of 10 μM 3,3′-T_2, and by 17% (n=9; NS) in the presence of 10 μM 3,5-T_2, whereas 10 μM Triac or Tetrac had no effect at all (both n=9; NS). After 24 h of incubation, the relative effect of 3,3′-T_2 was less (26%, n=9; P<0·02) than that observed after 15 min of incubation, whereas the effects of 3,5-T_2 (13%), Triac and Tetrac (both 19%) were still not significant. The effects of 10 μM unlabeled T_3 are given for comparison, and amounted to 52% (P<0·001) and 63% (P<0·001) after 15 min and 24 h respectively.

Presence of DITPA and T_3 during culture

A second approach to evaluate possible effects of DITPA on the T_3 uptake system was to add 10 nM DITPA during culture for 5 days and compare its effect with the presence

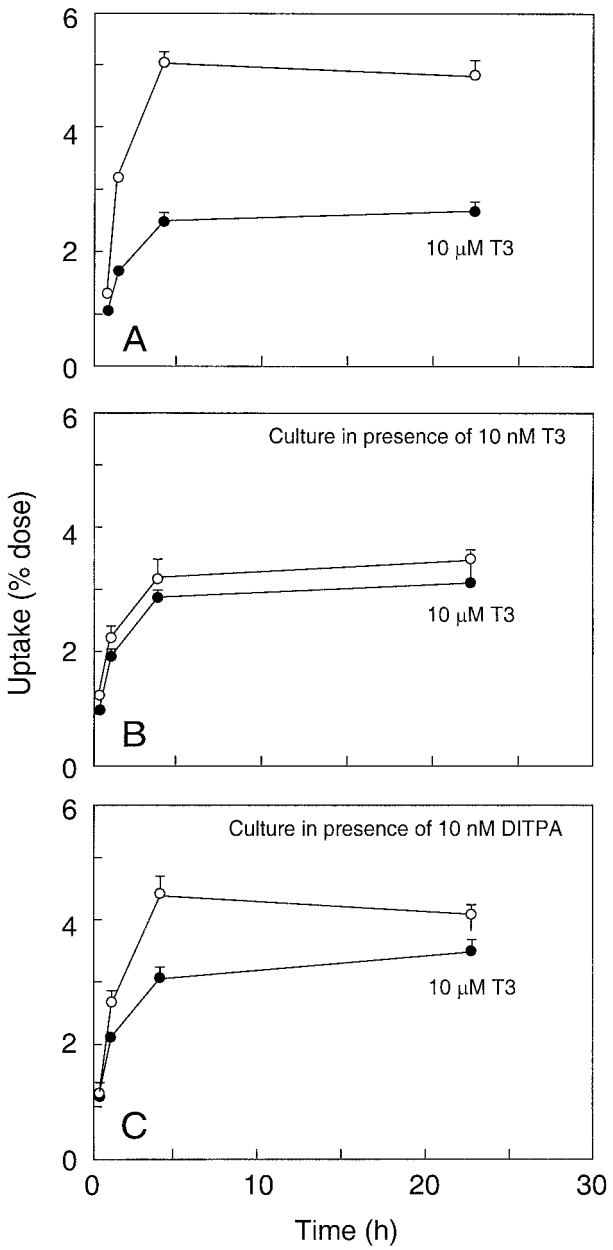

Figure 5 Time-course of [^{125}I]T_3 uptake in the absence (○) or presence (●) of 10 μM unlabeled T_3 in rat cardiomyocytes cultured without additions (A), with 10 nM T_3 present (B) or with 10 nM DITPA present (C). Data are expressed as % dose, and represent mean ± S.E. of six to 12 observations from two independent experiments.

of 10 nM T_3. This experiment was performed twice and, after culture, the time course of [^{125}I]T_3 uptake was tested for from 15 min to 24 h in the absence or presence of 10 μM unlabeled T_3 (Fig. 5). The time-course of [^{125}I]T_3 uptake in the presence of 10 μM unlabeled T_3 was about the same in the three culture conditions (compare Fig. 5A, B and C, lower curves). However, uptake of [^{125}I]T_3 without additions during incubation was significantly greater in cells that had been cultured under control conditions (Fig. 5A) than in cells that had been cultured in the presence of 10 nM T_3 (Fig. 5B) or DITPA (Fig. 5C). Whereas the absolute reduction in T_3 uptake by 10 μM unlabeled T_3 after 24 h of incubation was 2·01% of the dose (42% inhibition; $P<0·001$) in the cells cultured under control conditions, this was only 0·44% and 0·47% of the dose (12% inhibition; NS) in cells cultured in the presence of 10 nM T_3 or DITPA. The protein content per well under the different culture conditions was the same.

Discussion

In the present study, we explored further the uptake mechanism for T_3 in heart by comparing uptake of [^{125}I]T_3 with that of [^{125}I]Triac and by testing the effects of thyroid hormone metabolites and the analog DITPA on T_3 uptake in neonatal rat cardiomyocytes. Our previous studies showed that T_3 uptake in neonatal heart occurred by a temperature- and energy-dependent mechanism that was slightly dependent on the Na^+ gradient (Everts *et al.* 1996*b*, Van der Putten *et al.* 2001), and sensitive to Ca^{2+} blockers (Verhoeven *et al.* 2001), whereas results for T_4 uptake were less clear. The effects of the Ca^{2+} channel inhibitors were not secondary to inhibition of Ca^{2+} influx, suggesting interference with the putative T_3 carrier in the plasma membrane, rather than an energetic linkage to effects of T_3 on ion channels (Verhoeven *et al.* 2001).

Comparison of the time-courses of T_3 and Triac uptake in neonatal rat cardiomyocytes showed that, when expressed as fmol/pM free hormone, Triac uptake was about 30% greater than that of T_3, but the difference was not as large as in cultured pituitary cells, in which an almost twofold difference was found (Everts *et al.* 1994). When the 2-h cellular uptakes of T_3 and Triac were measured and expressed as percentages of the doses, a fivefold greater value for T_3 was observed, whereas the relative inhibition by 10 μM of the respective unlabeled hormone was roughly the same. Together, these findings indicate that Triac is taken up by cardiomyocytes and bound to their nuclear receptors, as in pituitary cells (Everts *et al.* 1994). In contrast to pituitary cells, in which T_3 uptake was inhibited by Triac and *vice versa* (Everts *et al.* 1994), the 15-min T_3 uptake in cardiomyocytes was not significantly inhibited by increasing concentrations of Triac. As T_3 and T_4 are scarcely metabolized by deiodination or conjugation in neonatal cultured cardiomyocytes within 24 h (Everts *et al.* 1996*b*), we extended the incubation periods to test the various analogs up to 24 h. However, even after 24 h of incubation, we could not detect any inhibitory effect of 10 μM Triac, suggesting different transport mechanisms for T_3 and Triac in heart. Addition of Triac to the diet of rats induced an increase in oxygen consumption and heart

rate, roughly to the same extent as addition of D-T_3 (Boyd & Oliver 1960). In contrast, neither Triac nor D-T_3 acutely stimulated Na^+ currents in cardiomyocytes (Huang *et al.* 1999). Tetrac 10 μM reduced neither the plasma membrane uptake of T_3 at 15 min, or its cellular uptake after 24 h. This is clearly at variance with the findings in pituitary cells, in which Triac and Tetrac inhibited [^{125}I]T_3 uptake over the range 15 min–4 h to the same extent as unlabeled T_3, and both suppressed thyrotropin releasing hormone (TRH)-stimulated TSH release (Everts *et al.* 1994, 1995). Tetrac has also been reported to be more effective than T_4 in suppressing TSH secretion *in vivo* in hypothyroid rats (Lameloise *et al.* 2001), whereas it was less effective in inducing cardiac hypertrophy. In our study, the inhibitory effect of T_4 (around 25%) on T_3 uptake was, if anything, greater than that of Tetrac, albeit not significantly so.

Of the two other metabolites tested, 3,3′-T_2 significantly reduced T_3 uptake in cardiomyocytes, the effect after 15 min (52% inhibition) being twice as large as that after 24 h (26% inhibition), suggesting that 3,3′-T_2 primarily interacted at the level of the plasma membrane. The other diiodothyronine, 3,5-T_2, had no effect on T_3 uptake in heart cells between 15 min and 24 h of incubation. Interestingly, 3,5-T_2 has been shown to be more effective than 3,3′-T_2 in suppressing TRH-induced TSH release in pituitary cells (Everts *et al.* 1995). It is also regarded as the important diiodothyronine with respect to (direct) stimulation of heat production in mitochondria (Goglia *et al.* 1999), and is just as effective as T_3 in acutely stimulating Na^+ currents in neonatal rat cardiomyocytes (Huang *et al.* 1999).

Because of its possible preferential effect on myocardial contractility, the analog DITPA has been used, with the purpose of improving heart function after infarction, but so far only in animal studies (Pennock *et al.* 1993, 2000, Morkin *et al.* 1996, Spooner *et al.* 1999). It has been shown that DITPA binds to bacterially expressed thyroid hormone receptors (Morkin *et al.* 1996). At the cellular level, DITPA induces sarcoplasmic reticulum Ca^{2+} transport and protein expression (Pennock *et al.* 2000), but interpretation of data on changes in myosin heavy-chain isoenzyme has been more complicated (Spooner *et al.* 1999). From this point of view, there remains a lack of understanding of how DITPA works (Spooner *et al.* 1999). In the present study, DITPA was tested for effects on T_3 uptake in cardiomyocytes. Up to a concentration of 10 μM, DITPA had a less clear effect on the 15-min T_3 uptake (20–28%) as compared with 10 μM T_3 itself (46–52%). In contrast, the effect of 10 μM DITPA after 4 h of incubation was at least as great (56%) as that of unlabeled T_3 (42–63%; compare Figs 2 and 5A), suggesting that DITPA can bind to the cytosolic or nuclear T_3 binding sites of the cardiomyocytes. When DITPA was added during culture at a concentration of 10 nM, its effect was comparable to that of the same concentration of T_3: the inhibitory effect of 10 μM T_3 on cellular T_3 uptake at 24 h was only 12%, whereas it was 42% in cells cultured under control conditions. As we have not tested whether DITPA interacts with the nuclear T_3 receptors, it is difficult to conclude whether DITPA primarily interferes with plasma membrane T_3 transport or with cytosolic or nuclear T_3 binding. To what extent the inhibitory effect of DITPA on T_3 uptake in the cardiomyocyte results in attenuation or stimulation of thyromimetic effects, for example on expression of the Na^+,K^+-ATPase isoforms, is currently being investigated.

The aromatic amino acid, tyrosine, the precursor of iodothyronines, and tryptophan share the same transport system (Christensen 1990). In two studies with cardiomyocytes, we reported that tryptophan in concentrations of 0·5 and 2 mM significantly reduced T_3 uptake (maximum response 30–45%; Everts *et al.* 1996*b*, Van der Putten *et al.* 2001). Moreover, the effects of tryptophan were additive to those of oligomycin, an inhibitor of oxidative phosphorylation, suggesting that at least two different transport systems may be responsible for T_3 uptake in neonatal heart (Van der Putten *et al.* 2001). One could be a transport system that is capable of transporting aromatic amino acids. Kragie (1994) and Hennemann *et al.* (2001) have reviewed the properties of thyroid hormone transport mechanisms in many cell types. We have previously compared uptake mechanisms for thyroid hormones in pituitary and liver, and postulated that regulation of the transport in these two tissues was different (Everts *et al.* 1996*a*). This difference could play a part in maintenance of the low serum T_3 and normal TSH concentrations in non-thyroidal illness and fasting (Everts *et al.* 1996*a*, Hennemann *et al.* 1998, 2001). When the present data are compared with the results obtained in pituitary cells (Everts *et al.* 1994, 1995), it seems that the transport mechanism for T_3 into the heart is different. Whether T_3 uptake in heart is similar or different as compared with that in liver (Everts *et al.* 1996*a*) remains to be established. This question is of relevance to the development of drugs with tissue-selective thyromimetic activity (Ichikawa *et al.* 2000, Trost *et al.* 2000).

Conclusion

3,3′-T_2, and to a lesser extent DITPA and T_4, inhibit plasma membrane T_3 uptake in the cardiomyocyte, whereas 3,5-T_2, Triac and Tetrac have no significant effects. In contrast, Triac itself is taken up by the cardiomyocyte, but probably through a transport mechanism other than that for T_3. Our current research plan includes investigation of whether exposure of cardiomyocytes to Triac and DITPA results in thyromimetic effects, and whether exposure to compounds that inhibit T_3 transport (in particular 3,3′-T_2) results in reduction of T_3-induced effects in the cardiomyocyte.

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