American Journal of Physiology – Heart and Circulatory Physiology

# Inhibitory effects of calcium channel blockers on thyroid hormone uptake in neonatal rat cardiomyocytes

**Frank A. Verhoeven, Ellis P. C. M. Moerings, Jos M. J. Lamers, Georg Hennemann, Theo J. Visser and Maria E. Everts**

*Am J Physiol Heart Circ Physiol* 281:1985-1991, 2001.

**You might find this additional information useful...**

This article cites 35 articles, 18 of which you can access free at:
http://ajpheart.physiology.org/cgi/content/full/281/5/H1985#BIBL

Medline items on this article's topics can be found at http://highwire.stanford.edu/lists/artbytopic.dtl
on the following topics:
Biochemistry .. Channel Blocking
Endocrinology .. Thyroid
Oncology .. Thyroid Hormones
Oncology .. Thyroxine
Oncology .. Triiodothyronine
Physiology .. Rats

Updated information and services including high-resolution figures, can be found at:
http://ajpheart.physiology.org/cgi/content/full/281/5/H1985

Additional material and information about ***AJP - Heart and Circulatory Physiology*** can be found at:
http://www.the-aps.org/publications/ajpheart

This information is current as of November 20, 2006 .

***AJP - Heart and Circulatory Physiology*** publishes original investigations on the physiology of the heart, blood vessels, and lymphatics, including experimental and theoretical studies of cardiovascular function at all levels of organization ranging from the intact animal to the cellular, subcellular, and molecular levels. It is published 12 times a year (monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991. Copyright © 2005 by the American Physiological Society. ISSN: 0363-6135, ESSN: 1522-1539. Visit our website at http://www.the-aps.org/.

Downloaded from ajpheart.physiology.org on November 20, 2006

American Journal of Physiology – Heart and Circulatory Physiology

*Am J Physiol Heart Circ Physiol* 281: H1985–H1991, 2001.

# Inhibitory effects of calcium channel blockers on thyroid hormone uptake in neonatal rat cardiomyocytes

FRANK A. VERHOEVEN,[1] ELLIS P. C. M. MOERINGS,[1] JOS M. J. LAMERS,[2] GEORG HENNEMANN,[1] THEO J. VISSER,[1] AND MARIA E. EVERTS[1,3]
*Departments of [1]Internal Medicine III and [2]Biochemistry, Erasmus University Medical School, 3000 DR Rotterdam; and [3]Department of Veterinary Anatomy and Physiology, Utrecht University, 3508 TD Utrecht, The Netherlands*

Received 18 September 2000; accepted in final form 16 April 2001

**Verhoeven, Frank A., Ellis P. C. M. Moerings, Jos M. J. Lamers, Georg Hennemann, Theo J. Visser, and Maria E. Everts.** Inhibitory effects of calcium channel blockers on thyroid hormone uptake in neonatal rat cardiomyocytes. *Am J Physiol Heart Circ Physiol* 281: H1985–H1991, 2001.—The effects of the $Ca^{2+}$ channel blockers verapamil, nifedipine, and diltiazem on triiodothyronine ($T_3$) and thyroxine ($T_4$) uptake were tested in cultured cardiomyocytes from 2-day-old rats. Experiments were performed at 37°C in medium with 0.5% BSA for $[^{125}I]T_3$ (100 pM) or 0.1% BSA for $[^{125}I]T_4$ (350 pM). The 15-min uptake of $[^{125}I]T_3$ was 0.124 ± 0.013 fmol/pM free $T_3$ ($n$ = 6); $[^{125}I]T_4$ uptake was 0.032 ± 0.003 fmol/pM free $T_4$ ($n$ = 12). Neither $T_3$ nor $T_4$ uptake was affected by 1% DMSO (diluent for nifedipine and verapamil). Uptake of $[^{125}I]T_3$ but not of $[^{125}I]T_4$ was dose dependently reduced by incubation with 1–100 μM verapamil (49–87%, $P < 0.05$) or nifedipine (53–81%, $P < 0.05$). The relative decline in $[^{125}I]T_3$ uptake after 4 h of incubation with 10 μM verapamil or nifedipine was less than after 15 min or 1 h, indicating that the major inhibitory effect of the $Ca^{2+}$ channel blockers occurred at the level of the plasma membrane. The reduction of nuclear $[^{125}I]T_3$ binding by 10 μM verapamil or nifedipine was proportional to the reduction of cellular $[^{125}I]T_3$ uptake. Diltiazem (1–100 μM) had no dose-dependent effect on $[^{125}I]T_3$ uptake but reduced $[^{125}I]T_4$ uptake by 45% ($P < 0.05$) at each concentration tested. Neither the presence of 20 mM $K^+$ nor the presence of low $Ca^{2+}$ in the medium affected $[^{125}I]T_3$ uptake. In conclusion, the inhibitory effects of $Ca^{2+}$ channel blockers on $T_3$ uptake in cardiomyocytes are not secondary to their effects on $Ca^{2+}$ influx but, rather, reflect interference with the putative $T_3$ carrier in the plasma membrane.

heart; culture; ATP; free hormone fraction

THYROID HORMONES induce a rise in cardiac output by their chronotropic and inotropic effects (23, 31). When thyroxine ($T_4$) is used in replacement therapy for hypothyroidism, this may induce myocardial ischemia and lead to angina pectoris (1). More recently, it has been shown that elevation of serum triiodothyronine ($T_3$) at hospital admission is associated with the development and progression of acute myocardial ischemia (22). Part of these effects may be secondary to the profound influence of thyroid hormones on $Ca^{2+}$ handling by the heart (5). In cardiomyocytes, *1*) $Ca^{2+}$ uptake by (L-type) voltage-gated channels in the plasma membrane is enhanced (14, 28), *2*) $Ca^{2+}$ uptake in the sarcoplasmic reticulum is accelerated (13, 18), and *3*) cellular $Ca^{2+}$ storage capacity is increased by thyroid hormone (13, 18).

$Ca^{2+}$ channel blockers are widely used in the treatment of angina pectoris and hypertension (19). Besides those conventional applications, $Ca^{2+}$ channel blockers have also been proposed as a supplement to $T_4$ treatment to control cardiac thyrotoxic symptoms (1, 17). $Ca^{2+}$ channel blockers act by inhibiting the $Ca^{2+}$ influx via the slow voltage-gated channels in the plasma membrane, thus leading to reduced contractility of vascular smooth muscle and the heart (19). This will result in a lower blood pressure and a decrease in myocardial oxygen consumption (5).

In myoblast (L6) cells, Topliss et al. (32) found that the uptake of $T_3$ was inhibited by organic $Ca^{2+}$ channel blockers in a dose-dependent fashion (0.1–100 μM) as follows: nifedipine > diltiazem > verapamil. In human Hep G2 hepatoma cells, 10 μM nifedipine inhibited $T_3$ uptake by ~80% (26). In neonatal cardiomyocytes, $T_3$ is taken up by an energy-dependent, carrier-mediated mechanism that may be partly dependent on the $Na^+$ gradient over the plasma membrane (10). For $T_4$, however, such a mechanism could not be demonstrated, although $T_4$ enters the intracellular compartment of the cardiomyocytes (10). In the perfused rat heart, $T_3$ had a much more pronounced inotropic effect than $T_4$ (27). Moreover, the same study showed that the inotropic effects of $T_3$ (1 nM–1 μM) could be blocked by nifedipine and verapamil (10 nM–10 μM).

The present study was undertaken *1*) to investigate the effects of organic $Ca^{2+}$ channel blockers on the uptake of $T_3$ and $T_4$ in cardiomyocytes and *2*) to explore the interaction between the uptake of thyroid hormones and changes in intra- and extracellular $Ca^{2+}$ concentration. This was done by testing the effects of

Address for reprint requests and other correspondence: M. E. Everts, Dept. of Veterinary Anatomy and Physiology, University of Utrecht, PO Box 80.157, 3508 TD Utrecht, The Netherlands (E-mail: m.everts@vet.uu.nl).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

 0363-6135/01 $5.00 Copyright © 2001 the American Physiological Society 

Downloaded from ajpheart.physiology.org on November 20, 2006

20 mM $K^+$ and low $Ca^{2+}$ in the incubation medium on $T_3$ uptake. In our experiments we have used a representative $Ca^{2+}$ channel blocker from each of the three structurally distinct classes of compounds: verapamil (a phenylalkylamine), nifedipine (a dihydropyridine), and diltiazem (a benzothiazepine).

## MATERIALS AND METHODS

*Cell culture.* Primary cultures of neonatal rat ventricular myocytes were prepared as described in detail previously (2, 36) with some modifications (10, 33). Briefly, hearts removed from 2-day-old Wistar rats were cut into small fragments 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 (HS), and 2% penicillin-streptomycin (PS) was then added to the suspended cells. This procedure was repeated seven times until most of the 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 of DMEM-M199 with 5% FCS, 5% HS, and 2% PS. The cell suspension was passed through nylon mesh, and the dispersed cells were preplated (2) into 250-ml culture flasks for 60 min (37°C, 5% $CO_2$) to remove fibroblasts. After this procedure, the preparation consists of >90% of cardiomyocytes (33). Cells of the enriched cardiomyocyte fraction were plated into 48-well culture dishes at subconfluent density ($5 \times 10^5$ cells/well) in 1-ml volumes of DMEM-M199 with 5% FCS, 5% HS, and 2% PS 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 fresh culture medium. Experiments were routinely performed after 5 days of culture.

*Cellular uptake of [$^{125}$I]$T_3$ and [$^{125}$I]$T_4$.* The incubation medium was identical to the culture medium, except serum was replaced by 0.5% bovine serum albumin (BSA) for measurements of [$^{125}$I]$T_3$ uptake and by 0.1% BSA for [$^{125}$I]$T_4$ uptake (10). After removal of the culture medium, cells were preincubated for 30 min at 37°C with 0.5 ml of medium without or with 1–100 μM verapamil, nifedipine, or diltiazem and then incubated in 0.25 ml of medium for 15–240 min at 37°C without or with the same additions plus [$^{125}$I]$T_3$ (100,000 cpm, 100 pM) or [$^{125}$I]$T_4$ (200,000 cpm, 350 pM).

After incubation, the medium was removed and the cells were washed with 1 ml of ice-cold saline to remove tracer not bound to the cells. Cells were dissolved in 1 ml of 0.1 N NaOH and counted for $^{125}$I activity in a 16-channel gamma counter (model NE 1600, Nuclear Enterprises, Edinburgh, Scotland). The amount of [$^{125}$I]$T_3$ or [$^{125}$I]$T_4$ 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 radioactivity recovered from the wells without cells.

To evaluate the effects of 20 mM $K^+$ or low $Ca^{2+}$ on [$^{125}$I]$T_3$ uptake, separate experiments were performed in Krebs-Ringer (KR) buffer containing 139 mM NaCl, 4.2 mM KCl, 1.5 mM $CaCl_2$, 1.4 mM $KH_2PO_4$, 1.4 mM $MgSO_4$, 5 mM D-glucose, 25 mM tris(hydroxymethyl)aminomethane (Tris), and 0.5% BSA (pH 7.4), where part of the NaCl (14.4 mM) was replaced by an equimolar amount of KCl or where $CaCl_2$ was omitted.

*Nuclear [$^{125}$I]$T_3$ binding.* Nuclear [$^{125}$I]$T_3$ binding was measured in cardiomyocytes cultured at a density of $10^6$ cells/well in 24-well culture dishes (10). Cells were preincubated for 30 min at 37°C with 0.5 ml of medium without or with 10 μM verapamil or nifedipine and incubated for 120 min at 37°C with 0.5 ml of medium with the same additions plus [$^{125}$I]$T_3$ (200,000 cpm, 100 pM) without or with 10 μM unlabeled $T_3$. After incubation, cells were washed once with 2 ml of ice-cold saline. The cells were scraped from the wells with a rubber policeman in 1 ml of PBS (on ice), and the wells were washed with 0.5 ml of PBS. These two aliquots were combined and centrifuged (300 *g*, 4°C, 7 min), and the cell pellet was counted (30 s) and solubilized in 1 ml of PBS containing 0.5% Triton X-100. After 2 min of continuous vortexing, nuclei were spun down (900 *g*, 4°C, 5 min) and washed once with 1 ml of PBS containing 0.5% Triton X-100. The nuclear pellets were counted for 5 min and frozen for DNA determination.

*Dilution of $Ca^{2+}$ channel blockers.* Stock solutions of verapamil and nifedipine (10 mM) were prepared in dimethylsulfoxide (DMSO) and further diluted in DMSO to obtain 1 and 0.1 mM. These solutions were diluted 100-fold in incubation medium to achieve final concentrations of 100, 10, and 1 μM verapamil or nifedipine. Diltiazem was dissolved in $H_2O$ and diluted similarly. Control incubations contained 1% DMSO for verapamil or nifedipine.

*Free $T_3$ and $T_4$ concentration.* Calculation of the free $T_3$ and $T_4$ concentration was based on determinations of the free fractions by equilibrium dialysis (30). Neither the free $T_3$ nor the free $T_4$ fraction was altered by the presence of 1% DMSO.

*ATP, DNA, and protein determinations.* The cellular ATP content was determined in 0.2 M perchloric acid extracts with the AEC kit (Lumac, Landgraaf, The Netherlands). DNA content was determined using a fluorometric method (8). The cellular protein content was determined with a protein assay kit (Bio-Rad, Munich, Germany). When cells were plated at a density of $5 \times 10^5$ cells/well, protein content after culture amounted to ~0.1 mg/well.

*Materials.* 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). Iodothyronines were obtained from Henning Berlin (Berlin, Germany). BSA (fraction V), diltiazem, nifedipine, and verapamil were purchased from Sigma Chemical (St. Louis, MO), DMSO and Tris from Merck (Darmstadt, Germany), 3′-[$^{125}$I]$T_3$ (3,070 μCi/μg) and 3′,5′-[$^{125}$I]$T_4$ (1,500 μCi/μg) from Amersham International (Aylesbury, UK), and Sephadex LH-20 from Pharmacia (Uppsala, Sweden). All other reagents were of the highest purity available.

*Validation of culture and washing procedure.* Although culture of cardiomyocytes in the presence of serum might result in proliferation of fibroblasts that also show active uptake of [$^{125}$I]$T_3$ and [$^{125}$I]$T_4$ (7), it has previously been shown that uptake of [$^{125}$I]$T_3$ and [$^{125}$I]$T_4$ was comparable in cardiomyocytes cultured in the absence and presence of serum (10). In the present study, this was repeated measuring [$^{125}$I]$T_3$ uptake in cells cultured for 5 days in the presence of 10% serum or 0.5% BSA or without any additions. The most regular and synchronous contractile activity over the whole well was observed in the serum-cultured cells, while the cells cultured in the other conditions presented irregular contractions. Values for [$^{125}$I]$T_3$ uptake differed <15% between the three conditions. Moreover, when the 5-min [$^{125}$I]$T_3$ uptake was compared in cardiomyocytes and fibroblasts in three independent experiments and expressed as percent dose per



American Journal of Physiology – Heart and Circulatory Physiology

Downloaded from ajpheart.physiology.org on November 20, 2006

microgram of protein, the following data were obtained: 0.066 ± 0.004 ($n$ = 3) and 0.006 ± 0.001 ($n$ = 3) for cardiomyocytes and fibroblasts, respectively, i.e., a 10-fold difference (S. M. van der Heide, unpublished results).

Sufficiency of the washing procedure after incubation was checked by a direct comparison of washing with saline alone and washing with saline containing 0.5% BSA. The 15-min uptakes of $[^{125}I]T_3$ and $[^{125}I]T_4$, expressed as percentage of the dose, were 0.60 ± 0.02 ($n$ = 3) and 0.13 ± 0.01 ($n$ = 3), respectively, with the usual washing procedure. With 0.5% BSA in the saline solution, these values amounted to 0.52 ± 0.04 ($n$ = 3) and 0.11 ± 0.01 ($n$ = 3), respectively.

*Calculations and statistics.* On the basis of the measurements of the free hormone fractions, the chemical concentration of the hormones in the incubation medium (100 and 350 pM for $[^{125}I]T_3$ and $[^{125}I]T_4$, respectively), and the incubation volume, the counts per minute were converted to femtomoles per picomolar free hormone.

The statistical significance of any of the observed effects was evaluated by Student's $t$-test or by one-way analysis of variance and Duncan's test for multiple comparisons. $P < 0.05$ was regarded as statistically significant.

## RESULTS

*Free $T_3$ and $T_4$ fraction.* The free $T_3$ fraction in medium with 0.5% BSA was ~4%, similar to the free $T_4$ fraction in medium with 0.1% BSA. Both were unaffected by the presence of 1% DMSO. The free $T_3$ and $T_4$ fractions showed no significant changes in the presence of 1–100 μM verapamil, nifedipine, or diltiazem. Similarly, the free $T_3$ fraction was unchanged by 20 mM $K^+$ or $Ca^{2+}$-free medium (data not shown).

*Time course of $[^{125}I]T_3$ uptake and nuclear $[^{125}I]T_3$ binding.* Figure 1 shows the time course of $[^{125}I]T_3$ uptake at 15 min, 1 h, and 4 h of incubation expressed as femtomoles per picomolar free $T_3$. $[^{125}I]T_3$ uptake was also measured in the presence of 10 μM verapamil, nifedipine, or diltiazem after preincubation for 30 min with these compounds. The uptake curve for $[^{125}I]T_3$ increased up to 4 h of incubation. Under control conditions, $[^{125}I]T_3$ uptake at 15 min, 1 h, and 4 h was 0.091 ± 0.004 ($n$ = 12), 0.246 ± 0.016 ($n$ = 6), and 0.399 ± 0.030 ($n$ = 6) fmol/pM free $T_3$, respectively. After 15 min, 1 h, and 4 h of incubation, $[^{125}I]T_3$ uptake was inhibited by 44% ($n$ = 9, $P < 0.05$), 46% ($n$ = 5, $P < 0.05$), and 25% ($n$ = 6, $P < 0.05$) in the presence of 10 μM verapamil and by 44% ($n$ = 9, $P < 0.05$), 56% ($n$ = 6, $P < 0.05$), and 38% ($n$ = 6, $P < 0.05$) in the presence of 10 μM nifedipine. In the presence of 10 μM diltiazem, $[^{125}I]T_3$ uptake after 1 h was reduced by 20% ($n$ = 6, $P < 0.05$) but showed no significant change after 15 min or 4 h of incubation.

It is important to note that the relative decline in $[^{125}I]T_3$ uptake after 4 h of incubation in the presence of 10 μM of the $Ca^{2+}$ channel blockers was less than that observed after 15 min or 1 h for verapamil and for nifedipine. This might imply that the major inhibitory effect of verapamil and nifedipine occurred at the level of the cardiomyocyte plasma membrane. Consequently, the amount of $[^{125}I]T_3$ bound to the nuclei should be proportionally decreased.

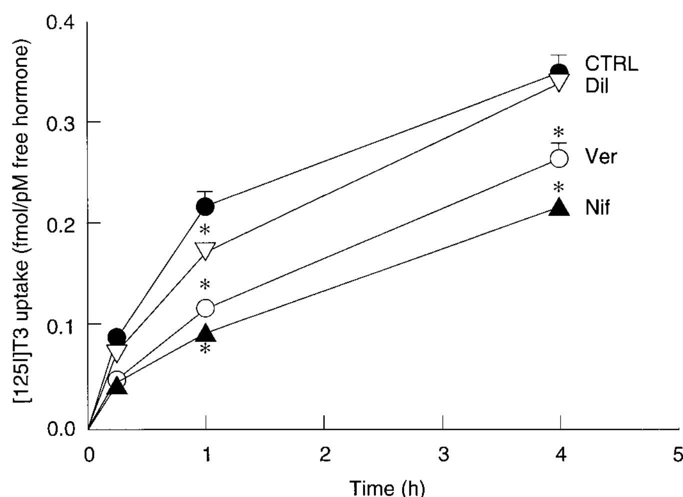


Fig. 1. Time course of uptake of $[^{125}I]$triiodothyronine ($T_3$) in cultured cardiomyocytes. Cardiomyocytes from 2-day-old rats were cultured at a density of ~5 × $10^5$ cells/well for 5 days in medium with 5% horse serum-5% FCS. Uptake of $[^{125}I]T_3$ (1 × $10^5$ cpm; 100 pM) was measured in medium with 0.5% BSA. Experiments were performed at 37°C with 30 min of preincubation and incubation times ranging from 15 min to 4 h in absence (control) or presence of 10 μM verapamil (Ver), nifedipine (Nif), or diltiazem (Dil). Values are means ± SE of triplicate observations from 2 experiments. *$P < 0.05$ vs. control.

This hypothesis was tested by incubating the cells for 2 h with $[^{125}I]T_3$ and verapamil or nifedipine, the most potent inhibitors of $T_3$ transport, isolating the nuclei, and determining DNA content. The results in Table 1 show that 10 μM unlabeled $T_3$, verapamil, or nifedipine equally reduced cellular $[^{125}I]T_3$ uptake by ~40%. This was associated with a reduction of nuclear $[^{125}I]T_3$ binding by 80% in the presence of 10 μM unlabeled $T_3$ and 44% in the presence of 10 μM verapamil or nifedipine. Consequently, the ratio of nuclear to cellular $[^{125}I]T_3$ uptake was 5% for cardiomyocytes incubated with unlabeled $T_3$ but was similar to control values (~15%) for cardiomyocytes incubated with verapamil or nifedipine. The DNA content of all nuclear pellets was the same.

*Plasma membrane uptake of $[^{125}I]T_3$ and $[^{125}I]T_4$.* Uptake of $[^{125}I]T_3$ and $[^{125}I]T_4$ at the level of the cardiomyocyte plasma membrane was evaluated during initial uptake at 15 min of incubation. Under control conditions, the uptake of $[^{125}I]T_3$, expressed as femtomoles per picomolar free hormone, amounted to 0.124 ± 0.013 ($n$ = 6), while that of $[^{125}I]T_4$ was 0.032 ± 0.003 ($n$ = 12), a fourfold difference ($P < 0.05$) as previously described (10). Because verapamil and nifedipine were dissolved in DMSO, 1% DMSO was added to the control incubations. With 1% DMSO, the 15-min uptake of $[^{125}I]T_3$ was 0.110 ± 0.010 ($n$ = 17) fmol/pM free $T_3$ and that of $[^{125}I]T_4$ was 0.036 ± 0.005 ($n$ = 19) fmol/pM free $T_4$, i.e., not different from the controls without DMSO (see above).

Figure 2 shows the effect of preincubation and incubation with 1–100 μM of verapamil, nifedipine, or diltiazem on the uptake of $[^{125}I]T_3$. $[^{125}I]T_3$ uptake decreased to 13% of control value ($n$ = 5, $P < 0.05$) with



American Journal of Physiology – Heart and Circulatory Physiology

Downloaded from ajpheart.physiology.org on November 20, 2006

Table 1. *Effects of unlabeled $T_3$, verapamil, and nifedipine on cellular and nuclear [$^{125}$I]$T_3$ activity, the ratio of nuclear to cellular [$^{125}$I]$T_3$ uptake, and DNA content of the nuclear pellet*

| Experimental Conditions | Cell Pellet (A) | Nuclear Pellet (B) | (B/A)* 100% | DNA Content, μg |
|---|---|---|---|---|
| Controls | 2.84 ± 0.31 | 0.43 ± 0.09 | 15.0 ± 1.8 | 1.38 ± 0.23 |
| $T_3$ (10 μM) | 1.78 ± 0.14* | 0.09 ± 0.01* | 5.1 ± 0.4* | 1.55 ± 0.06 |
| Verapamil (10 μM) | 1.69 ± 0.16* | 0.24 ± 0.05* | 14.1 ± 2.1 | 1.39 ± 0.15 |
| Nifedipine (10 μM) | 1.70 ± 0.27* | 0.24 ± 0.01* | 14.7 ± 2.5 | 1.61 ± 0.12 |

Values are means ± SE of triplicate observations of a representative experiment. 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 ~$10^6$ cells/well. After 1 day, culture medium was changed, and culture was continued for 4 days. Cells were incubated at 37°C for 2 h with [$^{125}$I]triiodothyronine ($T_3$, 200,000 cpm) in medium with 0.5% BSA. Cells were scraped from the wells in PBS and centrifuged, and the cell pellet was counted. The nuclear pellet was obtained after treatment with Triton X-100. *$P < 0.05$ vs. controls.

100 μM verapamil (Fig. 2*A*). With nifedipine, identical results were found (Fig. 2*B*). Although uptake of [$^{125}$I]$T_3$ was slightly increased to 133% ($n = 6$, not significant) in the presence of 1 μM diltiazem, it was 59% ($n = 12$, $P < 0.05$) and 75% ($n = 6$, not significant) of control with 10 and 100 μM diltiazem, respectively (Fig. 2*C*).

The effects of preincubation and incubation with 1, 10, or 100 μM verapamil, nifedipine, and diltiazem on the uptake of [$^{125}$I]$T_4$ are shown in Fig. 3. With verapamil, uptake of [$^{125}$I]$T_4$ was reduced only at the highest concentration (Fig. 3*A*). With nifedipine, [$^{125}$I]$T_4$ uptake remained at control levels (Fig. 3*B*). Finally, diltiazem inhibited uptake of [$^{125}$I]$T_4$ by ~45% independent of the concentration (Fig. 3*C*).

*ATP content.* The effects of the $Ca^{2+}$ channel blockers on the ATP content of the cardiomyocytes were investigated in two independent experiments. Compared with the $H_2O$ controls (100.0 ± 6.1%, $n = 6$), diltiazem (10 μM) did not alter the ATP content (104.8 ± 2.0%, $n = 6$), nor did 1% DMSO (81.2 ± 3.2%, $n = 6$), 10 μM verapamil in 1% DMSO (78.8 ± 8.3%, $n = 6$), or 10 μM nifedipine in 1% DMSO (95.2 ± 5.3%, $n = 6$).

*Effects of 20 mM $K^+$ and low $Ca^{2+}$ on [$^{125}$I]$T_3$ uptake.* Effects of the $Ca^{2+}$ channel blockers on [$^{125}$I]$T_3$ uptake could be secondary to their inhibitory effect on $Ca^{2+}$ influx into the cardiomyocyte or to interference of the $Ca^{2+}$ channel blockers with the $T_3$ transport system. To discriminate between these two possibilities, [$^{125}$I]$T_3$ uptake was measured under conditions known to increase (20 mM $K^+$) or reduce (low $Ca^{2+}$) the $Ca^{2+}$ concentration in the cardiomyocyte (16, 34). In skeletal muscle, we have demonstrated that the increase in intracellular $Ca^{2+}$ by 20 mM $K^+$ results from stimulation of the $Ca^{2+}$ influx that can be prevented by $Ca^{2+}$ channel blockers (9).

Because the concentration of cations cannot be changed in incubation medium based on DMEM-M199, these experiments were carried out in KR buffer, where $Na^+$ was partly replaced by $K^+$ or $Ca^{2+}$ was omitted. The results are shown in Fig. 4. In normal KR buffer, [$^{125}$I]$T_3$ uptake was 0.210 ± 0.004 fmol/pM free $T_3$ ($n = 23$), i.e., almost twice the value measured in DMEM-M199 (compare with Fig. 2). As shown in Fig. 4, 20 mM $K^+$ neither affected the uptake of [$^{125}$I]$T_3$ nor altered the inhibitory effects of 10 μM verapamil and nifedipine on [$^{125}$I]$T_3$ uptake. Furthermore, the effects of verapamil, nifedipine, and diltiazem were identical in KR buffer (Fig. 4) and DMEM-M199 (Fig. 2). In KR buffer without $Ca^{2+}$, uptake of [$^{125}$I]$T_3$ was 113 ± 7% of the control uptake ($n = 9$, not significant; not shown).

## DISCUSSION

The profound influence of thyroid hormone on heart function is well known (23, 31). Most of the effects of thyroid hormone on heart require transport of $T_3$ or $T_4$ across the plasma membrane, although acute cellular actions at the plasma membrane may be important as well, in particular in heart (6, 12, 15, 27, 35). In neonatal rat cardiomyocytes, a carrier-mediated uptake mechanism for $T_3$ similar to that in skeletal muscle (24) has been demonstrated (10). In myoblasts of the L6 cell line as well as in Hep G2 cells, $Ca^{2+}$ channel blockers were found to inhibit $T_3$ uptake (26, 32). In rat cardiac ventriculocytes, 100 μM verapamil was also found to reduce the efflux of $T_3$ (25).

Our results show that representatives from two different classes of organic $Ca^{2+}$ channel blockers, i.e., verapamil and nifedipine, reduced the uptake of [$^{125}$I]$T_3$ in cardiomyocytes in a dose-dependent fashion, whereas they had a less pronounced effect on the uptake of [$^{125}$I]$T_4$. Diltiazem, on the other hand, reduced neither $T_3$ nor $T_4$ uptake in a dose-dependent way. Only at a diltiazem concentration of 10 μM was a minor reduction of $T_3$ uptake seen, while $T_4$ uptake was inhibited to the same extent at 1, 10, or 100 μM diltiazem. The relative potency for inhibition of $T_3$ uptake in cardiomyocytes, i.e., nifedipine > verapamil > diltiazem, is consistent with their relative potency for inhibition of myocardial contractile force (29). Also qualitatively, the effects of nifedipine and verapamil on myocardial contractile function are different from those of diltiazem (3). It is interesting to note that 10 nM–10 μM nifedipine and verapamil inhibited the acute $T_3$-induced increase in cardiac inotropic activity of the perfused rat heart in a time- and dose-related fashion (27). This study showed that the acute effect of $T_3$ on the heart is $Ca^{2+}$ dependent. The results of our experiments as well as those of Topliss et al. (32) in isolated cells, however, suggest that the effect of the $Ca^{2+}$ channel blockers on $T_3$ uptake is not $Ca^{2+}$ dependent (see below). This discrepancy can be



American Journal of Physiology – Heart and Circulatory Physiology

Downloaded from ajpheart.physiology.org on November 20, 2006

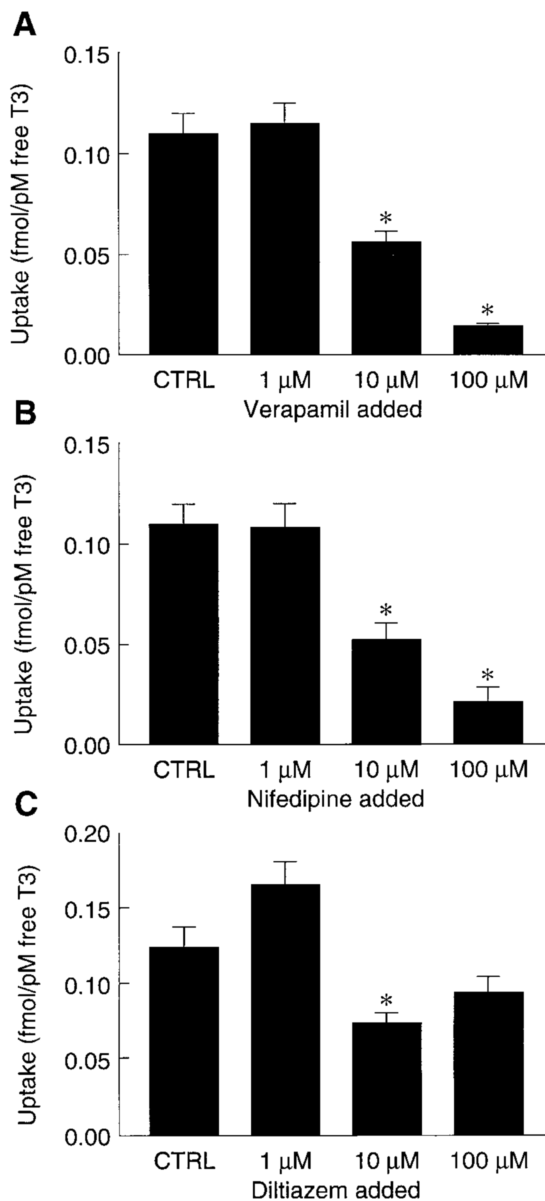


Fig. 2. Effects of Ca$^{2+}$ channel blockers on the 15-min uptake of [$^{125}$I]T$_3$. Cardiomyocytes from 2-day-old rats were isolated and cultured as described in Fig. 1 legend. Uptake of [$^{125}$I]T$_3$ ($1 \times 10^5$ cpm; 100 pM) was measured as described in Fig. 1 legend, with 30 min of preincubation and 15 min of incubation in absence or presence of 1, 10, or 100 µM verapamil (*A*), nifedipine (*B*), or diltiazem (*C*). Values are means ± SE of triplicate observations from 2–5 experiments. *$P < 0.05$ vs. control.

understood with the assumption that the Ca$^{2+}$-dependent inhibition of inotropy relates to a plasma membrane-mediated nongenomic effect of T$_3$ (27), while inhibition of T$_3$ uptake by Ca$^{2+}$ channel blockers will result in a reduction of intracellular T$_3$ and, thus, nuclear T$_3$ binding with potentially a subsequent reduction of the genomic effect.

T$_3$ uptake studies with rat L6 myoblasts showed inhibitory effects of Ca$^{2+}$ channel blockers in the following order, nifedipine > diltiazem > verapamil, at 1–100 µM in protein-free medium (32). As in cardiomyocytes, T$_3$ is actively taken up in skeletal muscle by an energy-dependent process (24). However, skeletal muscle Ca$^{2+}$ channels are not identical to those of cardiac muscle (19). Moreover, despite the fact that the density of Ca$^{2+}$ channel blocker-binding sites is ~100-fold greater in skeletal muscle than in cardiac muscle (19), skeletal muscle is relatively insensitive to Ca$^{2+}$ channel blockers (11, 19).

Previously, it was demonstrated by fluorometric (fura 2-AM) measurements of the intracellular free Ca$^{2+}$ concentration that incubation of neonatal rat cardiomyocytes with low Ca$^{2+}$ and high K$^+$ decreased and increased, respectively, the level of intracellular

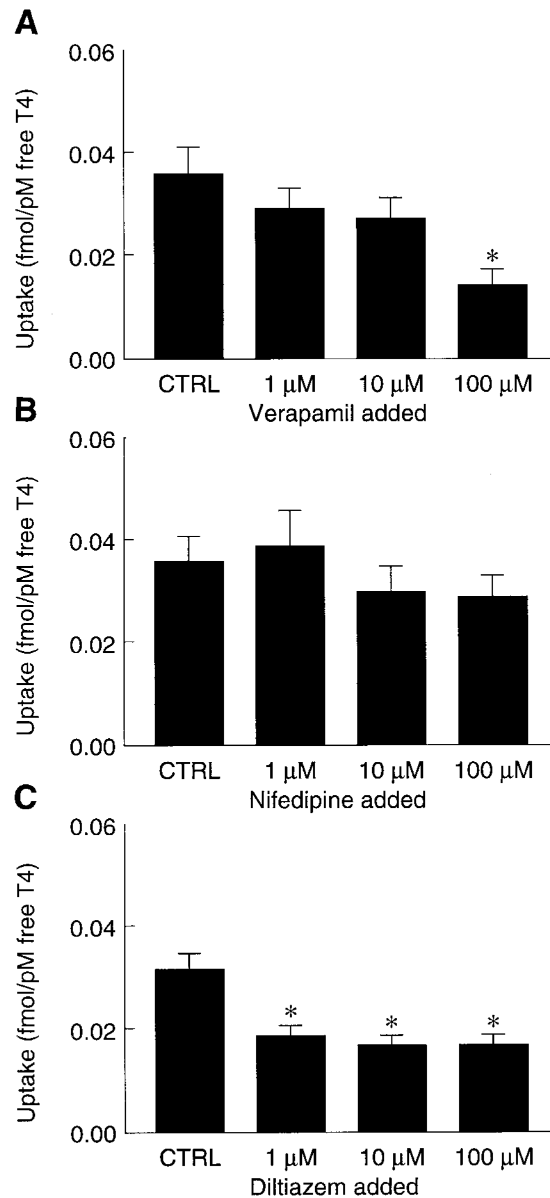


Fig. 3. Effects of Ca$^{2+}$ channel blockers on the 15-min uptake of [$^{125}$I]thyroxine (T$_4$). Cardiomyocytes from 2-day-old rats were isolated and cultured as described in Fig. 1 legend. Uptake of [$^{125}$I]T$_4$ ($2 \times 10^5$ cpm; 350 pM) was measured as described in Fig. 2 legend, in absence or presence of 1, 10, or 100 µM verapamil (*A*), nifedipine (*B*), or diltiazem (*C*) in medium with 0.1% BSA. Values are means ± SE of triplicate observations from 3–5 experiments. *$P < 0.05$ vs. control.



American Journal of Physiology – Heart and Circulatory Physiology
Downloaded from ajpheart.physiology.org on November 20, 2006

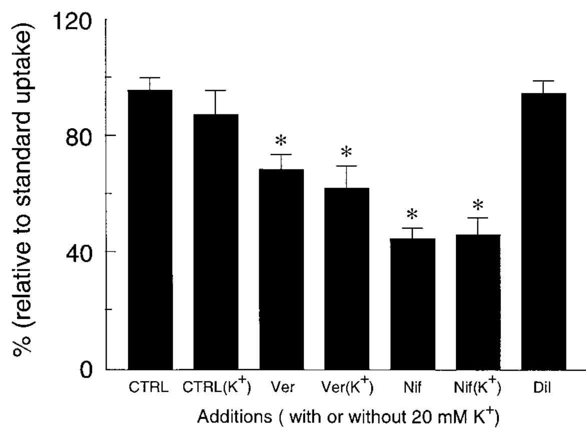


Fig. 4. Effects of 20 mM $K^+$ on $[^{125}I]T_3$ uptake in absence or presence of $Ca^{2+}$ channel blockers. Cardiomyocytes from 2-day-old rats were isolated and cultured as described in Fig. 1 legend. Uptake of $[^{125}I]T_3$ ($1 \times 10^5$ cpm; 100 pM) was measured as described in Fig. 2 legend in Krebs-Ringer buffer with 0.5% BSA in absence or presence of 10 μM verapamil, nifedipine, or diltiazem with 5.6 or 20 mM $K^+$ ($K^+$). Values are means ± SE of triplicate observations from 3 experiments. *$P < 0.05$ vs. control.

free $Ca^{2+}$ (34). Similar to results obtained in L6 myoblasts (32), our experiments performed in low-$Ca^{2+}$ medium or in medium containing 20 mM $K^+$ did not reveal any change in $T_3$ uptake. Furthermore, the effect of the $Ca^{2+}$ channel blockers on $[^{125}I]T_3$ uptake was not altered in the presence of 20 mM $K^+$. Therefore, from the present results it seems that inhibition or stimulation of the $Ca^{2+}$ flux itself (9) does not provide the basis for the inhibitory effects of the $Ca^{2+}$ channel blockers on $[^{125}I]T_3$ uptake. This leaves as alternative possibilities: *1*) a structural similarity between the $Ca^{2+}$ channel blocker-binding site and a thyroid hormone transporter binding site or *2*) a physicochemical similarity between the $Ca^{2+}$ channel blockers and $T_3$ itself. Finally, the question has been addressed as to whether the inhibitory effects of $Ca^{2+}$ channel blockers on $T_3$ uptake may be mediated by their calmodulin antagonism (32). Because the calmodulin antagonistic activity of most $Ca^{2+}$ channel blockers is very low (19), we have not further investigated this possibility.

When 10 μM unlabeled $T_3$ was added to incubations of cardiomyocytes with $[^{125}I]T_3$, the inhibitory effect was greater after 2–4 h than after 15 min of incubation (10). Moreover, the reduction of nuclear $[^{125}I]T_3$ binding was almost complete, while that of cellular $[^{125}I]T_3$ uptake was ~40% (10) (this study). The inhibitory effect of the $Ca^{2+}$ channel blockers was greater after 15 min than after 4 h of incubation, while the reduction of nuclear $[^{125}I]T_3$ binding by nifedipine and verapamil was proportional to the reduction of cellular $[^{125}I]T_3$ uptake. This suggests that $Ca^{2+}$ channel blockers do not directly interfere with nuclear $T_3$ binding and that the inhibition of $[^{125}I]T_3$ uptake by $Ca^{2+}$ channel blockers primarily occurs at the level of the plasma membrane. The data also show that the effect of $Ca^{2+}$ channel blockers on $[^{125}I]T_3$ uptake is not associated with changes in cellular ATP. This is important because the $T_3$ uptake mechanism in the cardiomyocyte is largely dependent on cellular ATP (10). Our finding of normal ATP contents at $Ca^{2+}$ channel blocker concentrations of 10 μM excludes a possible effect of any of the compounds via ATP.

Our preparation of cardiomyocytes cultured on culture dishes is not suited to study qualitatively or quantitatively the effects of short-term interventions on contractility of the cardiomyoctes. However, it is an important issue to address the effect of contractility per se on $T_3$ uptake. It has been shown that contractions of cardiomyocytes were smaller in medium with low $Ca^{2+}$ (16). Furthermore, exposure to 50 mM KCl (18) and 10 μM verapamil (4, 18) for 2–3 days resulted in contraction-arrested cells. Also, short-term incubation (20 min) with low $Ca^{2+}$, 50 mM KCl, or 1 μM nifedipine completely blocked contractility of the cardiomyocytes (34). However, the study by Muller et al. (18) demonstrated that the effect of 5 nM $T_3$ on the mRNA and the protein levels of the SERCA2 enzyme was identical in contracting and contraction-arrested cardiomyoctes, suggesting that $T_3$ can enter the cells and interact with the nucleus, independently of their contractile status. This confirmed the in vivo observation of Ojamaa et al. (21) that thyroid hormone could alter the expression of SERCA2 independently of the thyroid hormone-induced hemodynamic loading of the heart.

In human serum, the greater part of $Ca^{2+}$ channel blockers is bound to protein: ~90, 95, and 85% for verapamil, nifedipine, and diltiazem, respectively (29). Although the therapeutic levels of free $Ca^{2+}$ channel blockers in human serum are ~0.01 μM, in vitro effects are generally observed at free concentrations between 0.01 and 100 μM (29). Therefore, our experiments were performed using concentrations of 1–100 μM with 0.5 or 0.1% BSA in the medium, thus achieving free concentrations ranging from close to therapeutic levels to the concentrations used in in vitro experiments. Whereas the free concentration of the $Ca^{2+}$ channel blockers in experiments with $[^{125}I]T_4$ was higher because of the lower BSA concentration in the medium, the effects of nifedipine and verapamil on $[^{125}I]T_4$ uptake were smaller than those on $[^{125}I]T_3$ uptake. In view of the observations in our previous study (10) that $T_4$ entry into the cardiomyocyte is low and probably not mediated by an active transport mechanism, the effects of diltiazem or a high concentration of verapamil on $T_4$ uptake may be nonspecific and not indicative for inhibition of an active transport mechanism.

In summary, our study describes significant inhibitory effects of $Ca^{2+}$ channel blockers on the uptake of $T_3$ in cardiomyocytes and a less clear effect on the uptake of $T_4$. It has been shown that neither verapamil nor nifedipine directly affected the serum levels of free $T_3$, $T_4$, or thyroid-stimulating hormone (37). Our findings suggest that, in addition to the reduction of $Ca^{2+}$ entry into the cardiomyocyte, which suppresses myocardial contractile force and cardiac energy consumption (3, 5), $Ca^{2+}$ channel blockers will reduce the intracellular $T_3$ concentration in the cardiomyocyte. This



American Journal of Physiology – Heart and Circulatory Physiology

Downloaded from ajpheart.physiology.org on November 20, 2006

will contribute to a decrease in cardiac output and energy turnover and may thus help prevent myocardial ischemia. On the other hand, $T_3$ also acts on the smooth muscle cells, causing vascular relaxation, leading to a decrease in systemic vascular resistance (20). It is therefore important to realize that a $Ca^{2+}$ channel antagonist to be used in a clinical setting as a supplement to control cardiac thyrotoxic symptoms preferentially should have a greater effect on $Ca^{2+}$ influx in heart than in smooth muscle cells.

M. E. Everts held a fellowship of the Royal Netherlands Academy of Arts and Sciences. The study was supported by Netherlands Heart Foundation Grant 96.175.

## REFERENCES


1. **Bernstein R, Müller C, Midtbo K, Haug E, Nakken KF, Hertzenberg L, and Kjorstad KE.** Cardiac left ventricular function before and during early thyroxine treatment in severe hypothyroidism. *J Intern Med* 230: 493–500, 1991.
2. **Blondel B, Roijen I, and Cheneval JP.** Heart cells in culture: a simple method for increasing the proportion of myoblasts. *Experientia* 27: 356–358, 1971.
3. **Boucek, RJ Jr, Shelton M, Artman M, Mushlin PS, Starnes VA, and Olson RD.** Comparative effects of verapamil, nifedipine, and diltiazem on contractile function in the isolated immature and adult rabbit heart. *Pediatr Res* 18: 948–952, 1984.
4. **Cadre BM, Qi M, Eble DM, Shannon TR, Bers DM, and Samarel AM.** Cyclic stretch down-regulates calcium transporter gene expression in neonatal rat ventricular myocytes. *J Mol Cell Cardiol* 30: 2247–2259, 1998.
5. **Clausen T, Van Hardeveld C, and Everts ME.** Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol Rev* 71: 733–774, 1991.
6. **Davis PJ and Davis FB.** Acute cellular actions of thyroid hormone and myocardial function. *Ann Thorac Surg* 56: S16–S23, 1993.
7. **Docter R, Krenning EP, Bernard HF, and Hennemann G.** Active transport of iodothyronines into human cultured fibroblasts. *J Clin Endocrinol* 65: 624–628, 1987.
8. **Downs TR and Wilfinger WW.** Fluorometric quantification of DNA in cells and tissues. *Anal Biochem* 131: 538–547, 1983.
9. **Everts ME and Clausen T.** Effects of thyroid hormones on calcium contents and $^{45}$Ca-exchange in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 251: E258–E265, 1986.
10. **Everts ME, Verhoeven FA, Bezstarosti K, Moerings EPCM, Hennemann G, Visser TJ, and Lamers JMJ.** Uptake of thyroid hormones in neonatal rat cardiac myocytes. *Endocrinology* 137: 4235–4242, 1996.
11. **Godfraind T, Miller R, and Wibo M.** Calcium antagonism and calcium entry blockade. *Pharmacol Rev* 38: 321–416, 1986.
12. **Huang C-J, Geller HM, Green WL, and Craelius W.** Acute effects of thyroid hormone analogs on sodium currents in neonatal rat myocytes. *J Mol Cell Cardiol* 31: 881–889, 1999.
13. **Kim D and Smith TW.** Effects of thyroid hormone on $Ca^{2+}$ handling in cultured chick ventricular cells. *J Physiol (Lond)* 364: 131–149, 1985.
14. **Kim D, Smith TW, and Marsh JD.** Effect of thyroid hormone on slow $Ca^{2+}$ channel function in cultured chick ventricular cells. *J Clin Invest* 80: 88–94, 1987.
15. **Klemperer JD, Klein I, Gomez M, Helm RE, Ojamaa K, Thomas SJ, Isom OW, and Krieger K.** Thyroid hormone treatment after coronary bypass surgery. *N Engl J Med* 333: 1522–1527, 1995.
16. **McCall E, Ginsburg KS, Bassani RA, Shannon TR, Qi M, Samarel AM, and Bers DM.** Ca flux, contractility, and excitation-contraction coupling in hypertrophic rat ventricular myocytes. *Am J Physiol Heart Circ Physiol* 274: H1348–H1360, 1998.
17. **Milner MR, Gelman KM, Phillips RA, Fuster V, Davies TF, and Goldman ME.** Double-blind crossover trial of diltiazem versus propranolol in the management of thyrotoxic symptoms. *Pharmacotherapy* 10: 100–106, 1990.
18. **Muller A, Zuidwijk MJ, Simonides WS, and van Hardeveld C.** Modulation of SERCA2 expression by thyroid hormone and norepinephrine in cardiocytes: role of contractility. *Am J Physiol Heart Circ Physiol* 272: H1876–H1885, 1997.
19. **Nayler WG.** *Calcium Antagonists*. London: Academic, 1988, p. 69–85.
20. **Ojamaa K, Klemperer JD, and Klein I.** Acute effects of thyroid hormone on vascular smooth muscle. *Thyroid* 6: 505–512, 1996.
21. **Ojamaa K, Samarel AM, Kupfer JM, Hong C, and Klein I.** Thyroid hormone effects on cardiac gene expression independent of cardiac growth and protein synthesis. *Am J Physiol Endocrinol Metab* 263: E534–E540, 1992.
22. **Peters A, Ehlers M, Blank B, Exler D, Falk C, Kohlmann T, Fruehwald-Schultes B, Wellhoener P, Kerner W, and Fehm HL.** Excess triiodothyronine as a risk factor of coronary events. *Arch Intern Med* 160: 1993–1999, 2000.
23. **Polikar R, Burger AG, Scherrer U, and Nicod P.** The thyroid and the heart. *Circulation* 87: 1435–1441, 1993.
24. **Pontecorvi A and Robbins J.** Energy-dependent uptake of 3,5,3′-triiodo-L-thyronine in rat skeletal muscle. *Endocrinology* 119: 2755–2761, 1986.
25. **Ribeiro RCJ, Cavalieri RR, Lomri N, Rahmaoui CM, Baxter JD, and Scharschmidt BF.** Thyroid hormone export regulates cellular hormone content and response. *J Biol Chem* 271: 17147–17151, 1996.
26. **Scholz GH, Vieweg S, Uhlig M, Thormann M, Klossek P, Goldmann S, and Hofmann H-J.** Inhibition of thyroid hormone uptake by calcium antagonists of the dihydropyridine class. *J Med Chem* 40: 1530–1538, 1997.
27. **Segal J, Masalha S, Schwalb H, Merin G, Borman JB, and Uretzky G.** Acute effect of thyroid hormone in the rat heart: role of calcium. *J Endocrinol* 149: 73–80, 1996.
28. **Seppet EK, Kolar F, Dixon IMC, Hata T, and Dhalla NS.** Regulation of cardiac sarcolemmal $Ca^{2+}$ channels and $Ca^{2+}$ transporters by thyroid hormone. *Mol Cell Biochem* 129: 145–159, 1993.
29. **Singh BN and Opie LH.** *Drugs for the Heart*. Orlando, FL: Grune & Stratton, 1984, p. 39–64.
30. **Sterling K and Brenner MA.** Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. *J Clin Invest* 45: 153–163, 1966.
31. **Toft AD and Boon NA.** Thyroid disease and the heart. *Heart* 84: 455–460, 2000.
32. **Topliss DJ, Scholz GH, Kolliniatis E, Barlow JW, and Stockigt JR.** Influence of calmodulin antagonists and calcium channel blockers on triiodothyronine uptake by rat hepatoma and myoblast cell lines. *Metabolism* 42: 376–380, 1993.
33. **Van Heugten HAA, Bezstarosti K, and Lamers JMJ.** Endothelin-1 and phenylephrine-induced activation of the phosphoinositide cycle increases cell injury of cultured cardiomyocytes exposed to hypoxia/reoxygenation. *J Mol Cell Cardiol* 26: 1513–1524, 1994.
34. **Van Heugten HAA, De Jonge HW, Bezstarosti K, and Lamers JMJ.** Calcium and the endothelin-1 and $\alpha_1$-adrenergic stimulated phosphatidyl inositol cycle in cultured rat cardiomyocytes. *J Mol Cell Cardiol* 26: 1081–1093, 1994.
35. **Walker JD, Crawford FA Jr, Mukherjee R, and Spinale FG.** The direct effects of 3,5,3′-triiodo-L-thyronine ($T_3$) on myocyte contractile processes. *J Thorac Cardiovasc Surg* 110: 1369–1380, 1995.
36. **Yagev S, Heller M, and Pinson A.** Changes in cytoplasmic and lysosomal enzyme activities in cultured rat heart cells: the relationship to cell differentiation and cell population in culture. *In Vitro* 20: 893–898, 1984.
37. **Zofkova I, Blahos J, and Bednar J.** Effect of calcium antagonists on thyrotropin and thyroid hormone secretion. *Endokrinologie* 80: 231–234, 1982.




American Journal of Physiology – Heart and Circulatory Physiology

Downloaded from ajpheart.physiology.org on November 20, 2006