Effect of Thyroid Hormone on Slow Calcium Channel Function in Cultured Chick Ventricular Cells

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Abstract

The hyperthyroid state is associated with increased myocardial contractility. To clarify responsible mechanisms, we examined the effects of thyroid hormone on slow Ca channels, beta-adrenergic receptors, transsarcolemmal ^{45}Ca flux and cytosolic free calcium in cultured chick ventricular cells. Compared with cells grown without triiodothyronine (T_3), cells grown in 10 nM T_3 possessed (*a*) 67% ($P < 0.05$) more dihydropyridine ^{3}H-PN200-110 binding sites, (*b*) 24% ($P < 0.05$) more beta-adrenergic antagonist ^{3}H-CGP12177 binding sites, (*c*) a 57% ($P < 0.05$) greater nifedipine-sensitive initial ^{45}Ca uptake rate, and (*d*) a 31% ($P < 0.05$) greater nifedipine-sensitive ^{45}Ca uptake rate in response to BAY k 8644. Time-averaged mean intracellular free Ca concentration ($[Ca]_i$) measured with fura-2, total protein content, and dissociation constant values for ^{3}H-PN200-110 or ^{3}H-CGP12177 binding was not significantly different in the two groups of cells. BAY k 8644 (1 μM) increased mean $[Ca]_i$ 2.85- or 2.16-fold in cells grown with or without 10 nM T_3, respectively. *l*-Isoproterenol (1 μM) increased $[Ca]_i$ 1.53- or 1.28-fold in cells grown with or without 10 nM T_3, respectively. We conclude that thyroid hormone augments transsarcolemmal Ca influx, at least in part via slow Ca channels associated with increased numbers of these channels. T_3-treated cells appear to be more responsive to the effects of BAY k 8644 or isoproterenol on $[Ca]_i$.

Introduction

Hyperthyroidism is associated with increased heart rate, myocardial contractility, and cardiac output (1–3). Previous studies from our own and other laboratories suggest that thyroid hormone can act directly on heart cells, presumably via nuclear receptors, to produce an increase in contractile state as well as in protein synthesis (3–6). However, the cellular mechanisms by which thyroid hormone alters myocardial contractile state are not fully understood. Several studies have demonstrated the ability of thyroid hormone to increase myosin ATPase activity in the rat and rabbit by shifting the relative proportions of isoenzyme forms such that the form with higher ATPase activity (V1) predominates (3, 7, 8). These findings have led to the hypothesis that enhancement of myocardial contractility by thyroid hormones is the result of increased cardiac myosin ATPase activity. However, thyroid hormone has also been reported to alter Ca uptake by sarcoplasmic reticulum (9–14), to stimulate plasma membrane Ca-ATPase activity (4, 15, 16), and also to increase the numbers of sarcolemmal sodium pump sites (17–19). Therefore, the mechanisms by which myocardial contractility is enhanced in response to thyroid hormone appear to include altered Ca handling by sarcoplasmic reticulum and also changes in sarcolemmal membrane function that would directly influence the excitation-contraction coupling process.

We have previously reported that in monolayers of cultured chick embryo ventricular cells, thyroid hormone causes a time- and concentration-dependent increase in the rate of transsarcolemmal Ca movement and also in the size of the Ca pool associated with the sarcoplasmic reticulum (4). It is likely that the increased rate of inward Ca movement is partly responsible for enhanced myocardial contractility. To explore this issue further, in the present study we examined mechanisms that could potentially augment Ca entry. We tested the hypothesis that the increased rate of Ca influx produced by thyroid hormone is due to elevated numbers of slow Ca channels in the sarcolemmal membrane. In addition, we determined changes in ^{45}Ca uptake via nifedipine-sensitive pathways in response to growth in the presence of elevated levels of thyroid hormone as well as cytosolic free Ca concentrations in the presence or absence of the Ca channel agonist BAY k 8644 or the beta-adrenergic agonist isoproterenol.

Methods

Tissue culture. Monolayer cultures of spontaneously contracting chick embryo ventricular cells were prepared as described previously (20). Hearts of 10-d-old chick embryos were removed and individual ventricular cells were isolated by trypsinization. The final concentrations of K, Na, and Ca in culture media were 4.0, 137, and 0.97 mM respectively. The cell suspension was placed in plastic culture dishes containing 25-

monolayers were subsequently used in ion flux and contractility studies. Cultures were incubated in a humidified 5% CO_2, 95% air atmosphere at 37°C. Confluent monolayers, in which at least 80% of the cells exhibited spontaneous synchronous contraction, developed by 2–3 d of incubation.

After the cells were allowed to grow in culture medium containing 6% fetal calf serum for 48 h, the medium was replaced with serum-free medium containing 1 μM insulin, 50 nM transferrin, 1 nM selenium, 1 nM progesterone, and either no triiodothyronine (T_3)[1] or 10 nM T_3 (21). The final concentrations of K, Na, and Ca in the serum-free and serum-containing media were held constant. Cells continued to contract spontaneously and remained fully viable for at least 2 wk in the serum-free medium.

Receptor binding studies. The numbers of slow calcium channels and beta-adrenergic receptors in intact myocytes were estimated from

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1. *Abbreviations used in this paper:* $[Ca]_i$, intracellular free Ca concentration; T_3, triiodothyronine.

specific binding of the calcium channel antagonist ^{3}H-PN200-110 and the beta-adrenergic antagonist ^{3}H-CGP12177, respectively. ^{3}H-PN200-110 (Amersham Corp., Arlington Heights, IL) is a lipophilic dihydropyridine Ca channel antagonist with high affinity and specificity for the Ca channel (22, 23). ^{3}H-CGP12177 (Amersham Corp.) is a relatively hydrophilic beta-adrenergic antagonist that binds predominantly, if not exclusively, to cell surface beta-adrenergic receptors (24, 25). Cells were grown on six-well plates in the presence or absence of 10 nM T_3 for 48 h. Cells were washed with Hepes-buffered medium (pH 7.35) and then incubated with concentrations of ^{3}H-PN200-110 ranging from 100 to 1,400 pM, or ^{3}H-CGP12177 ranging from 50 to 2,000 pM for 30 min (steady state) at 37°C. Preliminary experiments have shown that in the concentrations used, at 37°C, association of PN200-110 with its binding site is complete by 15–20 min; CGP12177 binds with a similar time course. Binding was terminated by rapidly washing the cells in each well three times with 4 ml of cold Hepes-buffered medium (2–4°C). Cells were then solubilized with 1% sodium dodecyl sulfate (SDS) and 10 mM sodium borate. Radioactivity was assayed using a liquid scintillation spectrometer (60% efficiency; LKB Instruments Inc., Bromma, Sweden) and protein content was measured by the method of Lowry et al. (26). Nonspecific ^{3}H-PN200-110 or ^{3}H-CGP12177 binding observed in the presence of 10 nM nonlabeled PN200-110 or 1 μM *l*-propranolol, respectively, was subtracted from the total binding observed in its absence to calculate specific binding. Radioligand binding curves were analyzed with the iterative nonlinear least squares method of Munson and Rodbard (27) on a VAX 7800 computer to determine ligand affinity and number of binding sites.

Ca fluxes. For determination of Ca uptake by cultured chick ventricular cells, glass coverslips with attached monolayers were obtained from each culture. The coverslips were preincubated in Hepes-buffered medium (pH 7.35) containing 5 mM Hepes (*N*-2-hydroxyethylpiperazine, *N*-2-ethane sulphonic acid), 0.9 mM $CaCl_2$, 4 mM KCl, and 0.5 mM $MgCl_2$) for 10 min and then incubated in medium containing ^{45}Ca (5 μCi/ml). For studies using BAY k 8644, the ^{45}Ca uptake rate was determined by the following method. Cells were preincubated in Ca-free Hepes-buffered medium (37°C) for 5 min and then incubated in 0.9 mM Ca medium containing ^{45}Ca. BAY k 8644, when used, was present in both media. This extra step was necessary to ensure a known ^{45}Ca specific activity at the cell surface. Without this step, the apparent responses to BAY k 8644 or isoproterenol exposure were blunted, probably due to relatively slow mixing of ^{45}Ca with unlabeled Ca bound to sites at the cell surface. All incubations were at 37°C under conditions identical to those for the ligand binding studies. After the desired uptake period, coverslips were removed from the uptake medium and washed for 5 s each in three 80-ml vol of Hepes-buffered medium (4°C). This washing procedure has been shown to remove > 99% of ^{51}Cr after incubating the cells with the interstitial space marker ^{51}Cr-EDTA, and also to remove ^{45}Ca bound to the cell surface sites (20). Cells were then scraped from the coverslips and placed in 2 ml of 1% SDS, 10 mM sodium borate solution for 2 h to dissolve the cells. Aliquots of the medium containing dissolved cells were assayed for radioactivity in a scintillation spectrometer (LKB Instruments Co.) and for protein content. ^{45}Ca influxes were expressed in nanomoles per milligram protein. This provides a valid basis for comparison under our experimental conditions because T_3 did not alter significantly either the number of cells or protein content per plate during the 48-h exposure used in these studies (see below).

Cytosolic free Ca measurements. Intracellular free Ca concentration ($[Ca]_i$) was measured using the Ca-sensitive fluorescent dye fura-2. The permeant fura-2 AM enters the cell and the impermeant fura-2 produced as consequence of esterase action is trapped inside the cell. Cells grown on 12 × 30–mm rectangular glass coverslips were loaded with 2 μM fura-2 AM in culture medium for 20 min at 37°C. The cells were then washed for 20 min in Hepes-buffered medium to remove extracellular and bound dye. The glass coverslip was placed into a quartz cuvette which was then placed in the cuvette holder of a Fluorolog 2 fluorimeter (Spex Industries, Inc., Edison, NJ) equipped with magnetic stirrer and temperature control. Fura-2 fluorescence from the cells was continuously monitored at 505 nM emission wavelength. The dual excitation wavelengths were 340 and 380 nM. The relationship between fluorescence ratio (340 nm/380 nm) and $[Ca]_i$ was determined using the equations reported by Grynkiewicz et al. (28).

Contractility measurements. Changes in the contractile state of individual cells in the monolayers were assessed by the use of an optical-video system as previously described (20). After a 15-min equilibration period in serum-free medium, cells were superfused with desired test media, and the amplitude of cell motion and the first derivative of cell motion with respect to time were recorded in micrometers and micrometers per second, respectively.

Cell density correction. To normalize ion flux and content data for cell density on each coverslip, the monolayers were incubated in [^{3}H]leucine (0.2 Ci/ml) for 48 h before each experiment. ^{3}H and ^{45}Ca counts were simultaneously determined for each coverslip using standard double-label counting methods, allowing normalization of ^{45}Ca content relative to milligrams cell protein for each coverslip after the relationship of ^{3}H counts to protein content had been determined.

Miscellaneous. Insulin, transferrin, progesterone, and T_3 were purchased from Sigma Chemical Co., St. Louis, MO. Selenium ("Spectrapure") was purchased from United Mineral and Chemical Corp., New York, NY. Insulin-containing solutions were filtered through a Millix GV filter (Millipore Corp., Bedford, MA), pore size, 0.22 μM. BAY k 8644 was a gift from Miles Laboratories, Inc., New Haven, CT. Statistical analyses were performed using Student's *t* test (paired) or two-way analysis of variance.

Results

Effect of T_3 on the number of slow Ca channels. Ca movement across the cardiac sarcolemmal membrane and sarcoplasmic reticulum properties have been reported to be influenced by thyroid state (4, 11, 15). To test the hypothesis that altered Ca handling by the sarcolemmal membrane may involve a change in the number of slow Ca channels (and consequently Ca permeability) of the sarcolemmal membrane, we examined the effect of growth of cells with or without 10 nM T_3 on the number of slow Ca channels. Cultured chick ventricular cells were grown in serum-containing medium for 48 h and then grown in serum-free, chemically defined medium with or without 10 nM T_3 for an additional 48 h. The number of slow Ca channels in the plasma membrane of intact cells was then determined using ^{3}H-PN200-110, a potent Ca channel blocker. Compared with cells grown in the absence of T_3, cells grown in 10 nM T_3 had 67% more Ca channels as judged by specific binding of ^{3}H-PN200-110 (Table I; Fig. 1). T_3 had no statistically significant effect on ligand affinity. Because the total protein content, cell number per plate, and cell surface area values were not significantly different in the two groups of cells (see below), these results indicate that the number of slow Ca channels per cell increased significantly following 48 h exposure to T_3.

We then determined the number of beta-adrenergic receptors in the sarcolemmal membrane to determine whether growth in the presence of elevated thyroid hormone levels causes an increase in beta-adrenergic receptor number in cultured chick heart cells, as it does in cardiac muscle cells of other species (29–31). Using the hydrophilic ligand ^{3}H-CGP12177, a beta-adrenergic antagonist that selectively binds to cell surface beta receptors, we determined the number of ^{3}H-CGP12177 binding sites expressed on intact cells (Fig. 1). As summarized in Table I, cells grown in 10 nM T_3 bound 24% more ^{3}H-CGP12177 than cells grown without T_3. No significant change in the affinity of antagonist for beta-adrenergic receptors occurred. Therefore, the numbers of both slow Ca channels and beta-adrenergic receptors

Table I. Effect of T_3 on Numbers of Slow Calcium Channels and Beta-Adrenergic Receptors in Cultured Heart Cells

T_3	Number of slow Ca channels	K_d	Number of β-adrenergic receptors	K_d
	fmol/mg protein	*pM*	*fmol/mg protein*	*pM*
0	35.5±5.2	100±35	27.6±3.8	343±40
10 nM	59.4±4.9*	222±61	35.2±3.2*	348±62

Cells grown in medium with or without 10 nM T_3 were incubated with ^{3}H-PN200-110 or ^{3}H-CGP12177 and binding was allowed to reach steady state at 37°C (30 min). Binding was terminated by rapid washing of cells with cold Hepes-buffered solution. Cells were solubilized, and radioactivity and protein content in aliquots of dissolved cells were assayed. Specific binding for each ligand is the total binding minus nonspecific binding observed in the presence of nonlabeled ligands as described in Methods. Each value is the mean±SEM of five determinations.

* Significantly greater compared with the value observed in cells grown without T_3 ($P < 0.05$).

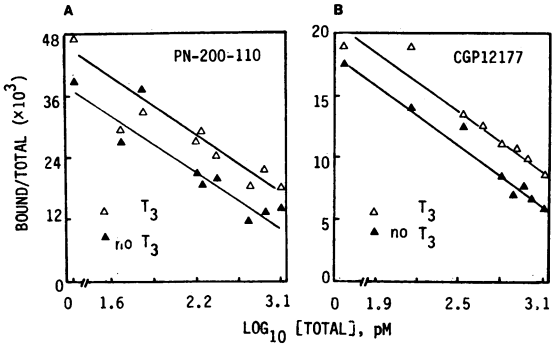

Figure 1. Binding of PN200-110 and of CGP12177 to intact cells. (*A*) ^{3}H-PN200-110 equilibrium binding was conducted in cells grown in 0 (*closed circles*) or 10 nM (*open circles*) T_3. Figure shows a representative experiment performed on parallel cultures simultaneously as described in Methods. This experiment was replicated five times with similar results. On average, there was no change in dissociation constant (K_d) for PN200-110 but a 67% increase in PN200-110 binding sites. (*B*) A representative ^{3}H-CGP12177 equilibrium binding experiment, as in *A*. On average, there was no significant change in K_d for CGP12177 but a 24% increase in receptor number for cells grown in 10 nM T_3.

are significantly increased by 48 h of exposure of cells to 10 nM T_3.

Effect of T_3 on cell function and growth. Thyroid hormone is well known to affect contractile function as well as growth of cardiac muscle cells. We have reported previously that growth of cultured chick embryo ventricular cells in 10 nM T_3 for 48 h increases the rate of spontaneous contractions and the velocity of cell motion compared with cells grown in the absence of T_3 (4). We examined these properties and also the growth properties of cells grown with or without 10 nM T_3 for 2–5 d. Growth of cells was studied by determining total protein content, numbers of cells per plate, and relative surface area of cells. Surface area was determined from the area of the two-dimensional image of the cells magnified 200-fold on the TV screen. Table II shows the results of these measurements. As reported earlier (19), the rate of beating and the velocity of cell motion were significantly increased in cells grown in 10 nM T_3 compared with values for cells grown in the absence of T_3. Mean total protein content per plate was not significantly different in the two groups of cells at day 1, 2, or 5. Surface area of the cells was also not measurably altered by 48-h growth in T_3. Because changes in the numbers of slow Ca channels and beta-adrenergic receptors occur without any corresponding change in cell size in cells grown with or without T_3, we conclude that T_3 increases the numbers of slow Ca channels and beta-adrenergic receptors per unit cell surface as well as per milligram protein.

Ca fluxes. We next determined whether alterations in the number of slow Ca channels are associated with corresponding changes in Ca flux via slow Ca channels. Cells grown in medium with or without 10 nM T_3 for 48 h were incubated in Hepes-buffered medium containing ^{45}Ca. The ^{45}Ca uptake by the cells was determined in the presence and absence of 10 μM nifedipine, a potent dihydropyridine Ca channel blocker. We specifically examined ^{45}Ca uptake over an interval of 10 s, a time during which ^{45}Ca flux is essentially unidirectional with minimal ^{45}Ca efflux. Nifedipine caused cessation of spontaneous beating in cultured chick heart cells grown with or without T_3. Therefore, a fraction of the decrease in ^{45}Ca uptake produced by nifedipine could be due to the absence of beating. However, nifedipine did not abolish action potentials during the 10 s of exposure, although action potential duration was shortened significantly (data not shown). Therefore, the difference in ^{45}Ca uptake observed in the presence and absence of nifedipine was taken as an indication of ^{45}Ca influx via slow Ca channels. The nifedipine-sensitive

Table II. Effect of T_3 on Contractile and Growth Properties of Cultured Heart Cells

T_3	Days in culture	Beating rate	Velocity of cell motion	Protein content	Surface area per cell
		per min	*μm/s*	*μg per plate*	*μm²*
0	2	106±6 (14)	30.6±3.3 (14)	282±21 (12)	18.6±5.4 (12)
10 nM	2	132±7 (14)*	39.1±4.2 (14)*	285±18 (12)	20.1±3.9 (12)
0	5	124±5 (12)	26.3±3.9 (12)	346±31 (12)	
10 nM	5	141±6 (12)*	33.2±2.7 (12)*	361±28 (12)	

Cells grown in the presence or absence of 10 nM T_3 for 48 h or 5 d were visualized using an optical-video system. Beating rates, velocity of cell motion, and surface area of the cells were determined. Protein contents were determined after 48 h or 5 d of growth. Values given are means±SEM; *n* values are given in parentheses. * Significantly greater compared with the value observed in cells grown without T_3 ($P < 0.05$).

^{45}Ca uptake was significantly greater (+57%; $P < 0.05$) in cells grown in 10 nM T_3 than in no T_3, indicating that the amount of Ca entering the cells per unit time was sensitive to thyroid hormone (Table III). The nifedipine-insensitive ^{45}Ca uptake was also significantly greater in cells grown with T_3 than without T_3. This is in agreement with our previous observations (4) and suggests that the pathway by which the increased nifedipine-insensitive Ca entry occurs in T_3-grown cells may be Na-Ca exchange.

The Ca channel agonist BAY k 8644 has been shown to increase Ca channel conductance in cardiac muscle cells (32, 33). We tested the hypothesis that cardiac myocytes grown in the presence of T_3 (and therefore having an increased number of slow Ca channels) have augmented Ca channel permeability changes in response to BAY k 8644. Cells were preincubated in Ca-free medium for 5 min and then incubated in Ca-containing medium with ^{45}Ca. Under the conditions of our experiments, 1 μM BAY k 8644 or 10 μM nifedipine produced maximal effects on ^{45}Ca uptake. Therefore, we used these concentrations of BAY k 8644 and nifedipine. As shown in Table III, addition of 1 μM BAY k 8644 to the preincubation and uptake media significantly augmented ^{45}Ca uptake in cells grown with or without T_3. Addition of 10 μM nifedipine to the media significantly decreased ^{45}Ca uptake compared with control and blocked the BAY k 8644-induced increase in ^{45}Ca uptake. The increase in the nifedipine-sensitive ^{45}Ca uptake rate produced by BAY k 8644 was significantly greater (+31%; $P < 0.05$) in cells grown in 10 nM T_3 (1.58 nmol/mg protein/min) than in cells grown without added T_3 (1.21 nmol/mg protein/min).

Table III. Effect of T_3 on ^{45}Ca Uptake

T_3	Drugs	^{45}Ca uptake	Nifedipine-sensitive ^{45}Ca uptake
		nmol/mg protein/min	*nmol/mg protein/min*
0		3.05±0.14	0.72±0.18
10 nM		3.98±0.18*	1.13±0.16*
0	Nifedipine	2.33±0.11	
10 nM	Nifedipine	2.85±0.12*	
0	BAY k 8644	5.46±0.21	1.21±0.17
10 nM	BAY k 8644	6.41±0.23*	1.58±0.11*
0	Nifedipine + BAY k 8644	4.25±0.31	
10 nM	Nifedipine + BAY k 8644	4.83±0.19*	

Cells grown in the presence or absence of 10 nM T_3 were incubated in Hepes-buffered medium, pH 7.35, containing ^{45}Ca with or without 10 μM nifedipine, 1 μM BAY k 8644, or nifedipine plus BAY k 8644 for 10 s. Experiments with BAY k 8644 were done using separate cultures (see Methods for detailed experimental procedures). Drugs were present in preincubation medium for 5 min before ^{45}Ca uptake experiments. Cells were then washed, dissolved, and assayed for ^{45}Ca and protein content. ^{45}Ca uptake is expressed as nmol/mg protein/min. Each value is the mean±SEM of eight determinations. Because the experimental procedures for ^{45}Ca uptake were different when using BAY k 8644, the uptake values on the bottom half of the table cannot be compared to those on the top half.

* Significantly greater than the value observed in cells grown in no T_3 ($P < 0.05$).

Cytosolic free Ca. Alterations in the number of slow Ca channels would be expected to be associated with changes in the cytosolic free Ca response to Ca channel agonist or beta-adrenergic agonist exposure. To test this hypothesis, we determined the effects of BAY k 8644 or isoproterenol on time-averaged $[Ca]_i$ in cells grown with or without 10 nM T_3. Fig. 2 shows the changes in fluorescence intensity observed in fura-2 loaded cells in response to BAY k 8644 and isoproterenol. Rapid exchange of Hepes-buffered medium (control) to that containing 1 μM BAY k 8644 or 1 μM isoproterenol produced a gradual increase in $[Ca]_i$ that reached a plateau by 4–5 min. This increase in $[Ca]_i$ was temporally related to the positive contractile response induced by BAY k 8644 or isoproterenol (see below). Nifedipine (10 μM) reduced $[Ca]_i$ and abolished the effects of BAY k 8644 or isoproterenol on $[Ca]_i$, as expected. The changes in $[Ca]_i$ produced by BAY k 8644 or isoproterenol in cells grown with or without T_3 are summarized in Table IV. In the absence of drugs, no significant differences in $[Ca]_i$ were noted in the two groups of cells. Because we measured the time-averaged $[Ca]_i$ and the beating rates are significantly different in cells grown in the presence and absence of T_3, comparisons of time-averaged $[Ca]_i$ values do not indicate how systolic and diastolic $[Ca]_i$ values may be affected. It is possible, however, to measure changes in mean $[Ca]_i$ produced by BAY k 8644 or isoproterenol. BAY k 8644 produced a 2.2- or a 2.9-fold increase, and isoproterenol produced a 1.3- or 1.5-fold increase in $[Ca]_i$ in cells grown in 0 or 10 nM T_3, respectively. Thus, both BAY k 8644 and isoproterenol caused a significantly greater increase in $[Ca]_i$ in cells exposed to elevated thyroid hormone concentrations ($P < 0.05$). These results indicate that an increase in the number of slow Ca channels is associated with an increased $[Ca]_i$ in response to interventions known to activate Ca channels. In the case of isoproterenol, the greater increase in $[Ca]_i$ observed in cells grown in T_3 is presumably due to a combination of increases in both beta-adrenergic receptors and slow Ca channels.

It has been reported that dihydropyridine Ca channel antagonists have a higher affinity for the inactivated (depolarized) state of Ca channels (34, 35). Because T_3 increases beating rate (4), we considered the possibility that the greater increase in $[Ca]_i$ produced by BAY k 8644 in T_3-grown cells compared with cells grown without T_3 was due to the difference in the rate of spontaneous beating. To test this hypothesis, cells grown without T_3 were equilibrated in either 3.5 or 5 mM K^+ medium (producing beating rates of 118±7 or 92±5, respectively), and the changes in $[Ca]_i$ in response to BAY k 8644 were determined. At steady state, BAY k 8644 increased $[Ca]_i$ from 126±21 to 244±26 nM in 3.5 mM K_0 medium and from 118±27 to 261±33 nM in 5 mM K^+ medium. Therefore, BAY k 8644-induced increases in $[Ca]_i$ were similar in both groups of cells despite the difference in beating rate, supporting the view that the larger increase in $[Ca]_i$ produced by BAY k 8644 in T_3-treated cells is due to factors other than beating rate. Although the beating rate increases in T_3-grown cells, the total fraction of time Ca channels spend in the depolarized state may not be appreciably different from that in cells grown without T_3, because the plateau phase of the action potential tends to become shorter with increasing beating rates.

Contractile state. To examine the relationship between the number of slow Ca channels and physiologic function, we de-

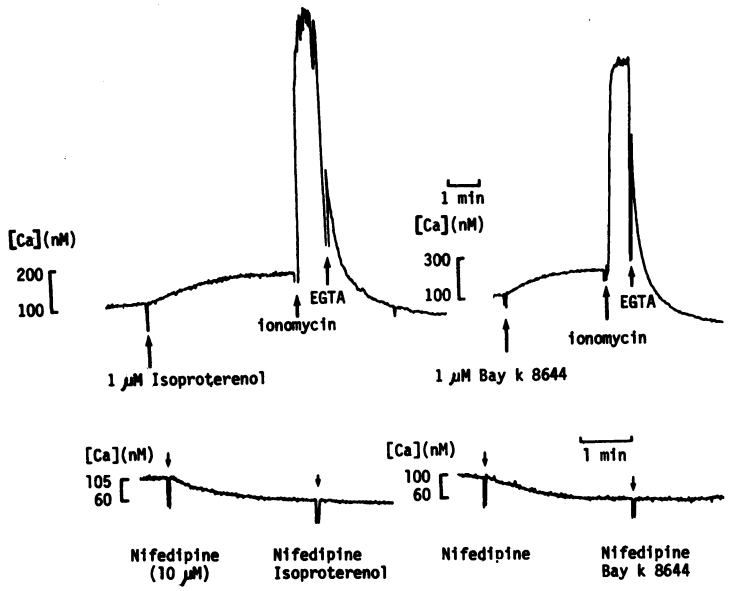

Figure 2. Effects of nifedipine, isoproterenol, and BAY k 8644 on $[Ca]_i$. Cells grown with or without 10 nM T_3 were loaded with fura-2 and then equilibrated in Hepes-buffered medium for 10 min. Fluorescence from the cells was continuously recorded. The cells were then exposed to medium containing 1 μM BAY k 8644 or 1 μM isoproterenol and changes in fluorescence intensity ratio (340 nm/380 nm) were monitored until a new steady-state level was reached. Fluorescence intensity ratio was calibrated to give actual [Ca] by the following method. When the steady-state level of fluorescence was reached, 4 μM ionomycin was added to the medium containing the cells to allow equilibration of intracellular and extracellular Ca. When the fluorescence reached the maximal level, EGTA (5 mM) was further added to reduce $[Ca]_i$ to near zero levels. The equation described by Grynkiewicz et al. (28) was then used to calculate [Ca] from the fluorescence intensities. The tracings shown below are obtained from cells grown in 10 nM T_3 for 48 h (n = 5).

termined the effect of BAY k 8644 on contractile state. Cells were perfused with control Hepes-buffered medium for 10 min and then with the same medium containing 1 μM BAY k 8644. BAY k 8644 produced a gradual increase in the amplitude of cell motion that reached steady state by 4–5 min. In each experiment, 3.6 mM Ca produced maximal increases in the amplitude and velocity of cell motion that were then taken as a 100% increase for comparison purposes. In cells grown in medium without T_3, BAY k 8644 increased the amplitude of cell motion by 51.4±5.7%. In cells grown in 10 nM T_3, BAY k 8644 increased the amplitude of cell motion by 59.2±5.6%. BAY k 8644 also augmented the velocity of cell motion in both groups of cells but no statistically significant differences in the increase were present ($P > 0.05$). Therefore, despite a greater increase in $[Ca]_i$ produced by BAY k 8644 in cells grown in 10 nM T_3, we could not detect a statistically significant difference in the augmentation of amplitude or velocity of cell motion in the two groups of cells.

Table IV. Effect of Isoproterenol and BAY k 8644 on $[Ca]_i$

T_3	Control	BAY k 8644		Isoproterenol	
	nM	*nM*		*nM*	
0	105.5±22.3	224.2±30.5*	‡	125.0±18.4*	‡
10 nM	102.0±22.4	286.5±30.9*		156.2±13.2*	

Cells grown with or without 10 nM T_3 were loaded with fura-2 and then equilibrated in Hepes-buffered medium (pH 7.35) for 10 min. Fluorescence from the cells was continuously monitored. The cells were then exposed to medium containing 1 μM BAY K 8644 or 1 μM isoproterenol, and changes in fluorescence intensities were monitored until a new steady state was reached. Fluorescence intensities were then calibrated to give actual [Ca] values (see legend to Fig. 1). Each value is the mean±SEM of five determinations.
* Significantly greater than the paired control value ($P < 0.05$).
‡ Significantly different ($P < 0.05$).

Discussion

The present study demonstrates that in cultured chick ventricular cells, thyroid hormone (T_3) modulates the expression of dihydropyridine binding sites, which very likely represent Ca channels in the sarcolemmal membrane. Analogous to the effects of T_3 on the numbers of sodium pump sites and beta-adrenergic receptors (17–19, 29, 30), cardiac myocytes grown in high levels of T_3 possessed greater numbers of dihydropyridine binding sites compared with cells grown without T_3. The increased number of binding sites was associated with an enhanced baseline contractile state and greater sarcolemmal Ca flux. There was also a greater increase in time-averaged $[Ca]_i$ in response to the Ca channel agonist BAY k 8644 and to isoproterenol. Taken together, these observations suggest that a hormone acting via a nuclear receptor (and presumably enhancing expression of a gene or group of genes) alters the expression of functional Ca channels.

Thyroid hormone–induced increases in myocardial contractility in the rat and rabbit have been attributed to changes in the proportions of V1 and V3 myosin isoenzymes, with the V1 isoenzyme form with higher ATPase activity increasing in response to thyroid hormone. Studies of isolated preparations of isolated sarcoplasmic reticulum from dog and rabbit hearts indicate that thyroid hormone also increases Ca uptake and loading of this organelle (9–14). Furthermore, thyroid hormone has been reported to stimulate sarcolemmal membrane Ca-ATPase (16). Thus, Ca movements across the membranes of the sarcolemma and sarcoplasmic reticulum of heart muscle appear to be altered by exposure to elevated thyroid hormone levels. We have shown previously that in cultured chick ventricular cells, unidirectional Ca flux rates across the sarcolemmal membrane as well as the size of the rapidly exchangeable Ca pool are increased in cells grown in the presence of T_3 compared with cells grown in its absence (4). The results of the present study indicate that the mechanism underlying the changes in transmembrane Ca transport involve increased slow channel Ca flux associated with increased dihydropyridine binding sites in

cells exposed to 10 nM T_3 for 48 h. The greater augmentation of slow channel Ca flux in response to BAY k 8644 in cells grown with T_3 is probably also related to an increased number of slow Ca channels in these cells. Studies with fura-2–loaded cells show that BAY k 8644 produces a significantly greater increase in time-averaged $[Ca]_i$ in cells grown with T_3 than without T_3, supporting the view that the increased numbers of dihydropyridine binding sites as judged by antagonist radioligand binding represent functional sites capable of mediating Ca entry.

Both PN200-110 and BAY k 8644 bind preferentially to the depolarized, largely inactivated state of the Ca channel. Bean (35) has shown that in canine ventricular cells, the affinity of a dihydropyridine for the calcium channel is more than 500-fold higher for the inactivated state of the channel than for the resting state. The T_3-grown cells have a somewhat higher beating rate and thus are in the inactivated, high-affinity state for BAY k 8644 more frequently and, hence, probably for a greater fraction of the time. This may account, in part, for the greater increase in $[Ca]_i$ in response to BAY k 8644 in cells grown in 10 nM T_3. However, in cells made to beat at mean rates of 92 or 118 per min by exposure to 5 or 3.5 mM K_0 medium, respectively, BAY k 8644 produced similar increases in $[Ca]_i$. This suggests that the significantly greater increase in $[Ca]_i$ produced by BAY k 8644 in T_3-grown cells compared with cells grown without T_3 is due to factors other than the small difference in beating rate.

Despite the greater increase in $[Ca]_i$ in response to BAY k 8644 in cells with higher numbers of dihydropyridine binding sites, the associated contractile response to the Ca channel agonist was not significantly different compared with that observed in cells with smaller numbers of slow Ca channels. We know of no precedent for decreased Ca sensitivity of contractile proteins in response to T_3. A more likely explanation for the similar percent increase in contractile amplitude response to BAY k 8644 in cells grown with or without T_3 may be that in the former cells, the basal velocity of cell contraction is closer to the maximal level than that of latter cells. It is expected that a cell that exhibits a lower level of force generation by the myofilaments will produce a greater percent increase in force when $[Ca]_i$ is increased than a cell starting from a higher level of force generation, at least for the concave-downward portion of the $[Ca]_i$-contractile effect curve.

In contrast to other studies showing that elevated thyroid hormone levels tend to cause hypertrophy (36, 37), total protein content (an estimate of cell growth) was not significantly different in our cultured chick embryo ventricular cell preparations during the first 48 h in the presence or absence of 10 nM T_3. Carter et al. (38) reported that in cultured chick heart cells, 10 nM T_3 caused a 16–21% increase in protein content during a 6-d exposure. In these studies, the increase in protein content after 48 h of T_3 treatment was significantly smaller than that observed after 6 d. The absence of a T_3 effect on protein content or on the size of the cells under the conditions of our experiments may be due to differences in growth media used. Conveniently, the lack of effect of T_3 on cell growth during the interval observed allows a direct comparison of Ca channel and beta-adrenergic receptor number as well as changes in Ca fluxes in T_3-treated and untreated cells, unencumbered by problems of normalization for cell size or considerations related to cell cycle in dividing myocytes. Even if a small difference in protein content were present, our data indicate that the changes in Ca channel and beta receptor number and $[Ca]_i$ observed in this study are of sufficient magnitude so that they could not be accounted for solely by small differences in protein content.

We have shown previously that in cultured chick heart cells, T_3 increases the number of Na pump sites in the cell membrane by 1.6-fold (19). Whether such changes are functionally related to the increased Ca uptake rate reported here is not clear. The available findings on the actions of T_3 do not suggest any specific relationship between an increased Ca uptake rate and the number of sodium pump sites. Because elevated numbers of Na pump sites were associated with reduced steady-state cellular Na content (19), the decreased $[Na]_i$ would be expected to be associated with reduced Ca influx via Na-Ca exchange. Because the ^{45}Ca uptake rate was significantly increased in T_3-treated cells despite the reduced $[Na]_i$, Ca uptake via pathways other than Na-Ca exchange is likely increased. This is in keeping with the results of the present study that growth in T_3 augments Ca uptake via slow Ca channels. It is also possible that growth in T_3 alters the number of Na-Ca exchangers or the affinity of the exchanger for Na and/or Ca, leading to increased Ca uptake via Na-Ca exchange. Our previous study (4) showing that Na-dependent Ca uptake was elevated in cells grown in T_3 compared with no T_3 supports the view that Ca influx via Na-Ca exchange may also be increased by growth in the presence of T_3.

In conclusion, thyroid hormone exposure increased the number of slow Ca channels and beta-adrenergic receptors in the sarcolemmal membrane of cultured chick heart cells. The increased myocardial contractility observed in the hyperthyroid heart appears to be due in part to increased numbers of slow Ca channels and, consequently, increased Ca influx via slow Ca channels. The Ca channel agonist, BAY k 8644, produced a greater increase in Ca uptake and $[Ca]_i$ in cells grown with than without T_3, further supporting the view that thyroid hormone modulates the number of functional Ca channels in the sarcolemmal membrane.

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