THE DIRECT EFFECTS OF 3,5,3'-TRIODO-L-THYRONINE (T₃) ON MYOCYTE CONTRACTILE PROCESSES

Insights into mechanisms of action

Administration of 3,5,3'-triiodo-L-thyronine (T₃) has recently been suggested to acutely improve left ventricular performance. However, the cellular and molecular mechanisms responsible for this improvement in left ventricular function with T₃ remained unknown. Accordingly, the present study examined the direct effects of T₃ administration on myocyte contractile function and the sarcolemmal systems that might potentially contribute to these effects. In isolated porcine left ventricular myocytes ($n = 81$), velocity of shortening increased in the presence of 80 pmol/L T₃ compared with that in untreated myocytes (117.0 ± 5.0 versus 77.3 ± 3.3 $\mu\text{m}/\text{sec}$, $p < 0.05$). In a separate series of experiments ($n = 29$), myocyte velocity of shortening increased in the presence of both T₃ and β -adrenergic receptor stimulation (25 nmol/L isoproterenol) to greater than that with β -adrenergic receptor stimulation alone (274.3 ± 16.9 versus 203.7 ± 16.2 $\mu\text{m}/\text{sec}$, $p < 0.05$). Cyclic adenosine monophosphate generation was next examined in isolated myocyte preparations ($n = 9$). In the presence of T₃, no significant increase in cyclic-adenosine monophosphate generation was observed compared with that in untreated myocytes (39.1 ± 8.3 versus 24.7 ± 5.8 fmols/myocyte, $p = 0.17$). However, in the presence of both T₃ and β -adrenergic receptor stimulation, cyclic-adenosine monophosphate generation increased significantly to greater than that with β -adrenergic receptor stimulation alone (224.4 ± 61.1 versus 120.1 ± 35.5 fmoles/myocyte, $p < 0.05$). Because cyclic-adenosine monophosphate modulates intracellular Ca⁺² processes, L-type Ca⁺² channel current (patch clamp methods; -picoamp/picofarad, $n = 15$) and peak intracellular Ca⁺² levels (fura 2 ionic measurement, $n = 47$) were next measured. In the presence of T₃, a shift in the activation voltage at peak L-type Ca⁺² channel current was observed from baseline (5.5 ± 1.4 versus 9.0 ± 1.0 mV, $p < 0.05$). Furthermore, in the presence of both T₃ and β -adrenergic receptor stimulation, peak L-type Ca⁺² channel current (8.9 ± 0.7 versus 6.3 ± 1.0 mV, $p < 0.05$) and peak intracellular Ca⁺² levels (189.9 ± 8.4 versus 171.7 ± 8.3 nmol/L, $p < 0.05$) increased compared with values obtained with β -adrenergic receptor stimulation alone. Important findings from the present study were twofold: (1) T₃ improved myocyte contractile processes through a cyclic-adenosine monophosphate-independent mechanism and (2) T₃ potentiated the effects of β -adrenergic receptor stimulation transduction by increasing cyclic-adenosine monophosphate production, L-type Ca⁺² channel current, and Ca⁺² availability to the myocyte contractile apparatus. Thus T₃ may provide a clinically useful adjunct to β -adrenergic agonist therapy. (J THORAC CARDIOVASC SURG 1995;110:1369-80)

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The active form of thyroid hormone, 3,5,3'-triiodo-L-thyronine (T_3), significantly contributes to cardiovascular performance.¹ A chronic elevation in the circulating level of T_3 causes an increase in heart rate and cardiac output and a decrease in systemic vascular resistance.²⁻⁵ In animal models, alterations in contractile protein expression and synthesis have been demonstrated to occur with persistent elevations in circulating T_3 levels.⁶⁻¹⁰ Finally, increased circulating levels of T_3 have been shown to augment oxygen consumption and increase rate of tension development in isolated papillary muscles.^{2, 11, 12} Although these effects of chronic elevation in the circulating levels of T_3 on cardiovascular performance are well established, the effects of a single acute administration of T_3 on fundamental contractile processes are incompletely understood. Past reports have suggested that acute administration of T_3 improves cardiac output after cardiopulmonary bypass.^{13, 14} Recently, this laboratory has demonstrated that acute administration of T_3 has direct and beneficial effects on isolated left ventricular (LV) myocyte contractile performance.^{15, 16} However, the underlying mechanisms for the beneficial effects of a single administration of T_3 on myocyte contractile function remain unknown. Accordingly, the overall goal of the present project was to determine the acute effects of T_3 on myocyte sarcolemmal processes and to relate these effects to changes in myocyte contractile function.

Past reports have demonstrated that elevation in the circulating level of T_3 can cause increased contractile protein messenger ribonucleic acid expression over a period of days.^{8, 9, 17-19} In a recent report from this laboratory, a 2-hour exposure to a single dose of T_3 improved myocyte contractile function and β -adrenergic responsiveness.¹⁶ Thus the acute and direct effects of T_3 on myocyte contractile function and β -adrenergic responsiveness that were observed in our recent studies were probably not caused by an alteration in contractile protein content. Several past reports have demonstrated that T_3 can directly modulate cell membrane enzyme systems.²⁰⁻²⁸ For example, the density and hydrolytic activity of the Na^+, K^+ -adenosinetriphosphatase (ATPase) system has been shown to be altered in the presence of T_3 .²⁶⁻²⁸ It has also been demonstrated that T_3 influences β -adrenergic receptor density and transduction.²¹⁻²³ Finally, T_3 regulation of intracellular Ca^{+2} by sarcolemmal Ca^{+2} -ATPase activity has been reported.²⁴ Taken together, these past studies suggest that T_3 may act

directly at the level of the myocyte sarcolemma. Therefore the present study was designed to test the hypothesis that the mechanism for the acute effects of T_3 on myocyte contractile function is a direct effect on myocyte sarcolemmal transduction systems.

Methods

Experimental design and rationale. The objective of the present study was to examine representative sarcolemmal systems that may be immediately affected by exposure to T_3 . The β -adrenergic receptor system located on the myocyte sarcolemma is an important mediator of intracellular events that directly influence myocyte contractile performance.^{29, 30} Activation of the β -adrenergic receptor system results in production of cyclic adenosine monophosphate (cAMP).^{29, 30} In light of the fact that T_3 may influence the activity of the β -adrenergic receptor system, cAMP production was measured in isolated myocytes at baseline and after T_3 administration, β -adrenergic receptor stimulation, or both.

Another important sarcolemmal system that modulates myocyte contractile performance is the Na^+, K^+ -ATPase system. This sarcolemmal receptor transports three Na^+ ions out of the myocyte and two K^+ ions into the myocyte for each ATP molecule hydrolyzed.^{30, 31} Previous reports have suggested that T_3 may influence Na^+, K^+ -ATPase hydrolytic activity.²⁶⁻²⁸ Accordingly, sarcolemmal preparations were used to measure the acute effect of T_3 on Na^+, K^+ -ATPase hydrolytic activity.

A third important sarcolemmal receptor system, the L-type Ca^{+2} channel, is responsible for triggering the release of intracellular Ca^{+2} within the cytoplasmic space of the myocyte. The L-type Ca^{+2} channel can be phosphorylated by cAMP-mediated events, which in turn can increase the contractile state of the myocyte.³² Previous reports from this laboratory demonstrated that in the presence of T_3 , myocyte β -adrenergic responsiveness was significantly increased.^{15, 16} This increased contractile response may be a result in part of augmentation of L-type Ca^{+2} channel current density. Accordingly, the present study examined the effects on L-type Ca^{+2} channel current density of acute treatment with T_3 , β -adrenergic receptor stimulation, or both.

A final common pathway that results from activation of the β -adrenergic receptor system, modulation of Na^+, K^+ -ATPase activity, or enhanced L-type Ca^{+2} channel current density is increased intracellular Ca^{+2} within the myocyte.²⁹⁻³² Therefore, in a final set of experiments, changes in intracellular Ca^{+2} content were directly measured in isolated myocytes after exposure to T_3 , β -adrenergic stimulation, or both.

Experimental design: myocyte isolation and contractile function measurement. Six pigs (Yorkshire strain, 28 kg) were anesthetized with isoflurane (0.5%/1.5 L per minute) and the lungs ventilated through a nonrecirculating anesthesia circuit. A sternotomy was done and the heart was quickly extirpated and placed in an oxygenated Krebs solution. The region of the LV free wall containing the left circumflex coronary artery was dissected free, the artery

was cannulated, and the tissue was prepared for myocyte isolation. All animals were treated and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.³³ With the use of methods described by this laboratory previously,³⁴⁻³⁶ Krebs solution containing collagenase (0.5 mg/ml, type II; 146 U/mg, Worthington, Pa.) was perfused through the circumflex artery. The tissue was then minced and triturated with 400 μ mol/L CaCl_2 and collagenase (0.5 mg/ml). After filtration of the supernatant, the cells were allowed to settle and the myocyte pellet was resuspended in standard culture medium (medium 199, nutrient mixture F-12, 2 mmol/L Ca^{2+} , Gibco Laboratories, Grand Island, N.Y.).

Myocyte contractile function was examined under the following conditions: (1) basal state (no T_3 , no isoproterenol), (2) in the presence of 80 pmol/L T_3 , (3) in the presence of 25 nmol/L isoproterenol, and (4) in the presence of both 80 pmol/L T_3 and 25 nmol/L isoproterenol. Video-assisted microscopy techniques described previously were used.³⁴⁻³⁶ The concentration of T_3 used in the present study was based on the results of previous dose-response studies done in this laboratory.¹⁵ T_3 was dissolved in NaOH and subsequently diluted to a concentration of 80 pmol/L T_3 with standard culture medium such that the final concentration of NaOH was 2×10^{-7} mol/L. This concentration of NaOH had no effect on myocyte contractile processes. The concentration of the nonselective β -adrenergic agonist, isoproterenol, was also chosen on the basis of findings of previous dose-response studies done in this laboratory.³⁷ Myocytes were imaged on an inverted microscope (Axiovert IM35, Zeiss Inc., Oberkochen, Germany) in a 2.5 cc tissue chamber with a thermoregulator to maintain the media temperature at 37° C. The oxygen tension of the medium was maintained at greater than 400 mm Hg throughout the experimental protocol. Myocytes were stimulated at 1 Hz and contractions were imaged with use of a charge-coupled device (GPCD60, Panasonic, Secaucus, N.J.). Myocyte motion signals were input through an edge detector system (Crescent Electronics, Sandy, Utah), converted into a voltage signal, digitized, and input to a computer (80286, ZBV2526, Zenith Data Systems, St. Joseph, Mich.) for subsequent analysis.³⁴⁻³⁶ Stimulated myocytes were allowed a 5-minute stabilization period after which contraction data for each myocyte were recorded from a minimum of 20 consecutive contractions. Parameters computed from the digitized contraction profiles included percentage shortening (percent), peak velocity of shortening (micrometers per second), peak velocity of lengthening (micrometers per second), total contraction duration (milliseconds), and time to peak contraction (milliseconds). Contractile measurements were obtained only on those myocytes that maintained a long-axis orientation perpendicular to the microscope objective throughout the contraction profile.

Measurement of cAMP content. Isolated myocytes (12,500 cells/0.5 ml) were incubated at 37° C in medium for 10 minutes under the following conditions: (1) basal state (no T_3 , no isoproterenol), (2) in the presence of 80 pmol/L T_3 , (3) in the presence of 25 nmol/L isoproterenol, and (4) in the presence of both 80 pmol/L T_3 and 25

nmol/L isoproterenol. Adenylate cyclase activity was determined by timed cAMP production with use of previously described methods.^{37,38} Reactions were terminated by placing the tubes in an ice-cold bath followed by centrifugation at 3250 g for 5 minutes. Pellets were resuspended in 0.5 ml buffer (50 mmol/L Tris-HCl, 10 mmol/L MgCl_2 , 10 μ mol/L egtazic acid, 10 μ mol/L phenylmethylsulfonylacid, 2.8 μ mol/L egtazic acid), boiled for 5 minutes, and then centrifuged at 6500 g for 10 minutes. The supernatant was assayed for cAMP content with use of a competitive radiolabeled assay (RIA Kit, Advanced Magnetics Inc., Cambridge, Mass.). Adenylate cyclase activity was determined at baseline, with T_3 , with β -adrenergic receptor stimulation, and in the presence of both T_3 and β -adrenergic receptor stimulation. Results were expressed as picomoles per liter cAMP produced/12,500 cells per 10 minutes. All measurements were done in duplicate.

Na^+ , K^+ -ATPase glycoside binding and hydrolytic activity. Sarcolemmal membranes were prepared from the harvested porcine LVs as previously described.^{37,39} The effect of T_3 on Na^+ , K^+ -ATPase hydrolytic capacity was examined by assessing p-nitrophenol-phosphatase (p-NPPase) activity.⁴⁰ Previously frozen LV myocardial samples were homogenized in 30 mmol/L histidine buffer (pH 7.4) at a 4:1 mixture (volume/weight). The assay was run at 37° C with use of a 215 μ l aliquot of tissue homogenate in a reaction medium containing (in final concentration) 150 mmol/L KCl, 20 mmol/L MgCl_2 , 30 mmol/L histidine (pH 7.4), 2 mmol/L egtazic acid, 10 mmol/L p-NPP (pH 7.4), and 3.3% bovine serum albumin. A standard curve was established with use of serial concentrations of p-nitrophenol read at 410 nm with a digital spectrophotometer (Spectronic 21D, Milton Roy Company, Belgium). Ouabain-sensitive p-NPPase activity was determined by adding 10 mmol/L ouabain to reaction mixtures and subtracting these results from those obtained in the reaction mixtures without ouabain. The reaction was terminated after 30 minutes by the addition of 0.1 ml of 50% trichloroacetic acid. Assays were run in duplicate and results were expressed as micrograms of p-nitrophenol released per milligram of protein per hour. Total protein concentration of tissue homogenates and sarcolemmal preparations were determined with the Bradford⁴¹ assay.

Measurement of L-type Ca^{+2} channel currents. Whole-cell L-type Ca^{+2} channel currents were measured by the whole-cell patch clamp method.⁴² Isolated myocytes were placed in a 0.5 ml Lucite acrylic resin tissue chamber mounted on the stage of an inverted microscope (Diaphot, Nikon Instruments, Garden City, N.Y.) and superfused with a modified Krebs's solution containing (in millimoles per liter): NaCl 133, KCl 4.7, dextrose 16.5, HEPES 20, MgCl_2 1.2, and CaCl_2 2.5 (pH 7.44). Microelectrodes (resistance 1.5 to 4 M Ω) were filled with pipette solution containing 100 μ g/ml nystatin (Sigma Chemical Co., St. Louis, Mo.).⁴³ After the formation of a gigaohm seal, when the patch was considered to be sufficiently permeabilized by nystatin, the superfusate solution was changed to an Na^+ - and K^+ -free solution that contained the following (in millimoles per liter): choline chloride 145, dextrose 5.5, MgCl_2 1.2, HEPES 5, CaCl_2 2.5, and 4-aminopyridine 2 (pH 7.2). All Ca^{+2}

Table 1. Acute effects of incubation with T_3 on myocyte function

	Steady state	Isoproterenol, 25 nmol/L
Percent shortening (%)		
No T_3	5.3 $\pm$ 0.2	10.4 $\pm$ 0.5*
80 pmol/L T_3	7.0 $\pm$ 0.3*	13.5 $\pm$ 0.5*†
Shortening velocity (μ m/sec)		
No T_3	77.1 $\pm$ 3.3	203.7 $\pm$ 16.2*
80 pmol/L T_3	117.0 $\pm$ 5.0†	274.3 $\pm$ 16.9*†
Lengthening velocity (μ m/sec)		
No T_3	86.2 $\pm$ 5.3	194.3 $\pm$ 17.8*
80 pmol/L T_3	130.8 $\pm$ 7.6†	260.3 $\pm$ 23.5*†
Time to peak contraction (msec)		
No T_3	232.1 $\pm$ 7.0	194.0 $\pm$ 9.7*
80 pmol/L T_3	215.6 $\pm$ 6.8	184.6 $\pm$ 6.5*
Duration of contraction (msec)		
No T_3	487.0 $\pm$ 12.6	425.5 $\pm$ 27.8*
80 pmol/L T_3	470.6 $\pm$ 11.2	427.4 $\pm$ 22.9*
No. of myocytes		
No T_3	81	29
80 pmol/L T_3	71	28

Values presented as mean plus or minus standard error of the mean.

* $p < 0.05$ versus steady state.

† $p < 0.05$ versus no T_3 .

channel currents were measured at room temperature (22° to 23° C) to enhance the temporal resolution of peak I_{Ca} measurements. Membrane voltage clamping protocols were generated with use of the pClamp software (version 5.6, Axon Instruments, Burlingame, Calif.) and a 12-bit digital-to-analog convertor (LabMaster TL-1, Axon Instruments). To examine the current-voltage (I-V) relationship for the L-type Ca^{+2} channels, the membrane holding potential was -40 mV.⁴² Clamp steps from -50 to +60 mV and 250 msec in duration were applied at 5-second intervals. Inward currents were normalized to membrane capacitance. Current signals were low-pass filtered at 5 kHz and led to a whole-cell patch clamp amplifier (Axopatch 200A, Axon Instruments). The analog current and voltage signals were digitized at 12-bit resolution (LabMaster TL-1, Axon Instruments) and the digitized data were stored on a computer (Z433Dh, Zenith Data Systems).

Parameters computed from the I-V relationship for peak L-type Ca^{+2} channel current included the maximum inward current and the membrane potential at which the Ca^{+2} channel current flow was maximal. The maximum inward current was computed to be the peak negative current from the I-V relationship. Measurements for each cell were made at baseline and in the presence of 80 pmol/L T_3 , of 25 nmol/L isoproterenol, or of both.

Measurement of intracellular Ca^{+2} . Isolated myocytes were resuspended in indicator-free medium (Dulbecco's modified Eagle's medium, Gibco Laboratories, Life Technologies, Inc., Grand Island N.Y.). Myocytes were studied under the following conditions: (1) basal state (no T_3 , no isoproterenol), (2) in the presence of 80 pmol/L T_3 , (3) in the presence of 25 nmol/L isoproterenol, and (4) in the presence of both 80 pmol/L T_3 and 25 nmol/L isopro-

enol. Ten microliters of fura 2/acetomethoxy ester (50 μ g fura 2, Molecular Probes, Inc., Eugene, Ore.; 50 μ l anhydrous dimethyl sulfoxide, Sigma) was added to the myocytes to yield a loading dye concentration of 5 μ mol/L. Myocytes were allowed to equilibrate for 1 to 2 hours in the dark before the experimental protocols were performed. Myocytes were studied in a thermostatically controlled chamber mounted on an Axiovert 35 inverted microscope (Zeiss). The microscope was interfaced to a spectrofluorometer and a digital fluorescence image analysis system, Attoflur Ratio Vision (version 6.0, Atto Instruments, Rockville, Md.). Fluorescence emissions were digitally collected at alternating excitation wavelengths of 340 and 380 nmol/L. At the end of each experiment, background autofluorescence from the myocytes was digitally subtracted. The background signal was less than 1.5% of the total fluorescent signal from the fura 2-loaded myocyte. The yield of viable myocytes after fura 2 loading was 75% $\pm$ 5%. Basal, peak, and timed averaged, steady-state intracellular Ca^{+2} measurements were recorded with a stimulation frequency of 30 pulses/min. The wavelength ratio was converted to an absolute Ca^{+2} level with use of precalibrated analytical standards.⁴⁴

Data analysis. Indices of myocyte contractile function for the groups treated with and without T_3 were compared by analysis of variance. For the β -adrenergic response studies, measurements at baseline and after β -adrenergic stimulation were directly compared by paired t test. In vitro assays for cAMP generation and L-type Ca^{+2} channel current density were compared for the treatment groups in a similar manner. Na^+, K^+ -ATPase hydrolytic activity was analyzed by t test. For intracellular Ca^{+2} measurements, a running average of 15 delivered stimuli was analyzed for each treatment by analysis of variance. After analysis of variance, mean separation was done with use of Bonferroni bounds.⁴⁵ All statistical procedures were done with the BMDP statistical software package (BMDP Statistical Software Inc., Los Angeles, Calif.). Results are presented as mean plus or minus the standard error of the mean. Values of $p < 0.05$ were considered to be statistically significant.

Results

Myocyte contractile function. In an initial series of experiments, the acute effects of T_3 on myocyte contractile function were examined (Table 1). In the presence of T_3 , myocyte percent shortening increased by 32% and myocyte velocity of shortening increased by 52%. In the presence of the β -adrenergic agonist isoproterenol, myocyte velocity of shortening increased by 164% ($p < 0.05$). More important, β -adrenergic stimulation after the addition of T_3 increased myocyte velocity of shortening to values higher than those for isoproterenol alone. Specifically, velocity of shortening in the presence of both T_3 and β -adrenergic receptor stimulation increased by an additional 34% compared with that with β -adrenergic stimulation alone ($p < 0.05$).

Thus, consistent with recent reports from this laboratory, T_3 improved indices of myocyte contractile function and β -adrenergic responsiveness.^{15,16} To determine whether T_3 exerted its acute influence through binding to the β -adrenergic receptor with resultant cAMP production, we examined myocyte contractile function after the addition of propranolol (a nonselective β -adrenergic; 25 nmol/L). The dose of propranolol used was sufficient to inhibit the contractile response of the myocyte to isoproterenol (data not shown). This concentration of propranolol had no effect on the increase in myocyte contractile function after the acute addition of T_3 . Thus the increase in contractile function with T_3 was apparently not mediated solely through binding to the β -adrenergic receptor. In the next series of experiments, potential contributory mechanisms for the effects of T_3 on myocyte contractile function were examined.

cAMP generation. The focus of this series of experiments was to examine whether T_3 influenced cAMP generation in isolated myocytes. The results from this series of experiments are summarized in Fig. 1. In the presence of T_3 alone, cAMP generation increased from baseline (no T_3) values (39 ± 8 fmols/myocyte versus 25 ± 6 fmols/myocyte, respectively), although this increase did not reach statistical significance ($p = 0.17$). However, cAMP generation increased to 120 ± 35 fmols/myocyte ($p < 0.05$) after β -adrenergic receptor stimulation. Furthermore, in the presence of both T_3 and β -adrenergic receptor stimulation, cAMP production increased to 224 ± 61 fmols/myocyte, which was significantly higher than values with β -adrenergic receptor stimulation alone ($p < 0.05$). Thus T_3 significantly augmented cAMP generation after β -adrenergic receptor stimulation in isolated myocytes.

Na^+, K^+ -ATPase hydrolytic activity. To determine whether T_3 modulated global myocyte sarcolemmal systems, Na^+, K^+ -ATPase hydrolytic activity was measured with use of purified LV sarcolemmal preparations. Basal Na^+, K^+ -ATPase hydrolytic activity was 2.2 ± 0.1 μ g/mg of protein per hour. This value is consistent with results previously obtained by this laboratory with similar LV sarcolemmal preparations.⁴⁰ Na^+, K^+ -ATPase hydrolytic activity did not increase in the presence of 80 pmol/L T_3 (2.3 ± 0.1 μ g/mg of protein per hour, $p = 0.62$). Therefore, in these studies of isolated LV sarcolemmal preparations, the acute addition of T_3 did not directly influence Na^+, K^+ -ATPase hydrolytic activity.

Fig. 1. cAMP generation in normal, isolated myocytes (no T_3), in presence of 80 pmol/L T_3 , with β -adrenergic receptor stimulation with isoproterenol (25 nmol/L), and in presence of both T_3 and β -adrenergic receptor stimulation. In presence of T_3 , cAMP generation was not significantly different from that in untreated myocytes. cAMP production increased significantly with β -adrenergic receptor stimulation. Finally, in presence of both T_3 and β -adrenergic receptor stimulation, cAMP production increased significantly higher than values observed with β -adrenergic receptor stimulation alone. Absolute values for cAMP generation are reported in Results section. * $p < 0.05$ versus no T_3 ; # $p < 0.05$ versus isoproterenol.

L-type Ca^{+2} channel currents. L-type Ca^{+2} channel current and voltage relationships were successfully obtained with use of perforated nystatin patch clamp techniques in 15 isolated myocytes. The I-V relationships for the L-type Ca^{+2} channel current at baseline (no T_3), with T_3 alone, with β -adrenergic receptor stimulation, and in the presence of both T_3 and β -adrenergic receptor stimulation are shown in Fig. 2. The peak L-type Ca^{+2} channel current computed for these conditions is summarized in Fig. 3. Although T_3 did not significantly increase L-type Ca^{+2} channel current density, the voltage at which peak Ca^{+2} channel current was obtained significantly shifted from the baseline value (5.5 ± 1.4 mV versus 9.0 ± 1.0 mV, $p < 0.05$). However, no difference in the voltage at peak current was ob-

Fig. 2. L-type Ca^{+2} channel I-V relationships obtained from 15 isolated myocytes with use of patch clamp microelectrode techniques. In presence of 80 pmol/L T_3 alone there was no apparent increase in L-type Ca^{+2} channel current, but significant leftward shift was observed for voltage at which peak current occurred. β -Adrenergic receptor stimulation with isoproterenol (25 nmol/L) caused change in I-V relationship. Specifically, significant increase in maximum L-type Ca^{+2} channel current density was observed. In presence of both T_3 and β -adrenergic receptor stimulation, further increase in L-type Ca^{+2} channel current density was observed. Absolute values for L-type Ca^{+2} channel current density are shown in Fig. 3. Voltages at which peak L-type Ca^{+2} channel current occurred are reported in Results section.

served after β -adrenergic receptor stimulation alone or in the presence of both T_3 and β -adrenergic receptor stimulation (6.0 ± 2.9 mV versus 5.6 ± 2.4 mV, respectively, $p = 0.14$). With β -adrenergic receptor stimulation, peak intracellular L-type Ca^{+2} channel current density increased significantly from baseline values. Furthermore, in the presence of both T_3 and β -adrenergic receptor stimulation, an additional increase in L-type Ca^{+2} channel current density was observed that was significantly higher than that obtained by β -adrenergic stimulation alone ($p < 0.05$). Therefore in the presence of T_3 maximum L-type Ca^{+2} channel current density was unchanged, but a significant shift occurred in the voltage at which peak current was obtained. Furthermore, T_3 augmented the L-type Ca^{+2} channel

Fig. 3. Peak intracellular L-type Ca^{+2} channel current density ($-\text{pA/pF}$) for isolated myocytes. No change in L-type Ca^{+2} channel current density occurred in presence of 80 pmol/L T_3 . Significant increase in L-type Ca^{+2} channel current density occurred after β -adrenergic receptor stimulation with isoproterenol (25 nmol/L). More important, in presence of both T_3 and β -adrenergic receptor stimulation, L-type Ca^{+2} channel current density increased to values higher than those with β -adrenergic receptor stimulation alone. * $p < 0.05$ versus no T_3 ; # $p < 0.05$ versus isoproterenol.

current density with concomitant β -adrenergic receptor stimulation.

Intracellular Ca^{+2} measurements. In a final series of experiments, myocyte intracellular Ca^{+2} was measured at baseline, with T_3 , with β -adrenergic receptor stimulation, and in the presence of both T_3 and β -adrenergic receptor stimulation. Intracellular Ca^{+2} measurements were first obtained in myocytes in the quiescent resting state (no electrical stimulation). Next, myocytes were stimulated at 0.5 Hz and steady-state and peak intracellular Ca^{+2} levels were determined. The results from this portion of the study are summarized in Table II. In normal myocytes (no T_3), both steady-state and peak intracellular Ca^{+2} levels significantly increased from values in the quiescent resting state after initiation of electrical stimulation ($p < 0.05$). In the presence of T_3 , steady-state and peak intracellular Ca^{+2} levels in-

Table II. Acute effects of incubation with T_3 on myocyte intracellular calcium levels

	Quiescent state	Electrical stimulation (0.5 Hz)	
		Steady state	Peak level
Calcium level (nmol/L)			
No T_3	110.2 $\pm$ 2.8	136.1 $\pm$ 3.1	159.2 $\pm$ 3.9
80 pmol/L T_3 alone	106.3 $\pm$ 3.6	135.1 $\pm$ 4.2	159.6 $\pm$ 5.7
25 nmol/L isoproterenol alone	109.4 $\pm$ 4.8	142.7 $\pm$ 7.4	171.7 $\pm$ 8.3*
80 pmol/L T_3 plus 25 nmol/L isoproterenol	117.2 $\pm$ 4.7	158.5 $\pm$ 7.5*	189.9 $\pm$ 8.4*†
No. of myocytes			
No T_3	47	47	47
25 nmol/L isoproterenol	18	18	18
80 pmol/L T_3	23	23	23
80 pmol/L T_3 plus 25 nmol/L isoproterenol	19	19	19

Values presented as mean plus or minus standard error of mean.

* $p < 0.05$ versus no T_3 .

† $p < 0.05$ versus isoproterenol.

creased from quiescent resting state values, but these values were not different from those in untreated myocytes. After β -adrenergic receptor stimulation, peak intracellular Ca^{+2} levels were significantly greater than peak intracellular Ca^{+2} levels in untreated myocytes ($p < 0.05$). Furthermore, peak intracellular Ca^{+2} levels were significantly higher in the presence of both T_3 and β -adrenergic receptor stimulation compared with those with β -adrenergic receptor stimulation alone ($p < 0.05$). Thus T_3 significantly augmented intracellular Ca^{+2} levels with concomitant β -adrenergic receptor stimulation.

Discussion

Past reports have suggested that the acute administration of T_3 may improve indices of LV pump performance and myocyte contractile function.^{13-16, 46-51} However, the contributory cellular and molecular mechanisms responsible for these acute effects of T_3 on LV and myocyte function remain incompletely understood. Accordingly, the present study investigated whether T_3 would influence fundamental determinants of myocyte contractile performance. Specifically, sarcolemmal transduction systems, intracellular cAMP generation, and intracellular Ca^{+2} levels were examined in the presence of T_3 . The important and unique findings from the present study were twofold. First, T_3 caused a significant shift in the activation voltage required for maximum L-type Ca^{+2} channel current density. Thus this leftward shift in the activation voltage of the L-type Ca^{+2} channel may have prolonged channel opening periods and thereby enhanced myocyte contractile processes. In the presence of both T_3 and β -adrenergic receptor stimulation, intracellular cAMP gen-

eration was increased, L-type Ca^{+2} channel current density was enhanced and myocyte peak intracellular Ca^{+2} levels were elevated. Consequently, one contributory mechanism for the beneficial effects of T_3 after β -adrenergic receptor stimulation may be a result of phosphorylation of the L-type Ca^{+2} channel with a subsequent increase in Ca^{+2} availability to the contractile apparatus. Therefore the present study provides unique evidence to suggest that T_3 has specific and selective effects on steady-state myocyte ionic homeostasis and intracellular events after β -adrenergic receptor stimulation.

The direct effects of acute administration of T_3 on LV pump performance have been the focus of several recent investigations.^{13, 14, 46-51} For example, Dyke and colleagues⁵⁰ demonstrated that T_3 improved LV peak developed pressure after global ischemia in isolated rabbit heart preparations. Moreover, Wechsler and colleagues⁴⁸ demonstrated that T_3 administration significantly improved the preload recruitable stroke-work relationship in pigs after cardiopulmonary bypass. In patients, it has been reported that acute administration of T_3 at the termination of cardiopulmonary bypass results in a decreased requirement for inotropic therapy.¹³ The present study, as well as a recent report,¹⁵ demonstrated that T_3 has direct effects on myocyte contractile performance. The present study builds on these past studies by identifying the potential mechanisms responsible for the beneficial effects of T_3 on LV and myocyte function. Specifically, in the presence of T_3 , a shift in the activation voltage at peak current for the L-type Ca^{+2} channel was observed. Further, in the presence of both T_3 and β -adrenergic receptor stimulation an increase was observed in

L-type Ca^{+2} channel current density, cAMP production, and intracellular Ca^{+2} level within the myocyte.

The classic mechanism of action for T_3 has been described to occur through the interaction with nuclear receptors and subsequent modifications in protein expression and translation.⁶⁻¹⁰ However, in the present study, as well as in past reports, the effects of T_3 have been observed to have an immediate onset of action with respect to LV and myocyte performance. Therefore an alternative mechanism for the acute actions of T_3 may be through modulation in the activity of myocyte sarcolemmal systems.²⁰⁻²⁵ Previous reports have shown that T_3 increased Na^+ channel activity, enhanced the transport of small molecules, and augmented sarcolemmal Ca^{+2} -ATPase activity.^{20, 24, 25, 52} For example, Rudinger and associates²⁴ reported an increase in basal Ca^{+2} -ATPase activity in normal rabbit sarcolemma in the presence of T_3 . In addition, Craelius, Green, and Harris⁵² reported an acute increase in the peak influx of Na^+ and increased duration of the action potential in a rat myocyte preparation after the addition of T_3 . These past reports provide evidence to suggest that T_3 directly modulates myocyte sarcolemmal function.²⁰⁻²⁵ The present study demonstrated that T_3 directly influenced specific sarcolemmal receptor systems, which included the L-type Ca^{+2} channel and the β -adrenergic receptor system. Therefore the present study provides evidence to suggest that the alterations in LV and myocyte function after T_3 administration were a direct result of modulation of myocyte sarcolemmal systems.

A common method to improve contractile state is through stimulation of the β -adrenergic receptor system. After binding of an agonist to the β -adrenergic receptor, a cascade of events occurs that includes modulation of a G protein complex, stimulation of adenylate cyclase, and subsequent production of cAMP.^{29, 30} The mechanisms of action for cAMP within the myocyte include phosphorylation of the sarcolemmal L-type Ca^{+2} channel and phospholamban on the sarcoplasmic reticulum.^{29, 30} Thus cAMP is an important determinant of intracellular Ca^{+2} availability to the contractile apparatus within the myocyte. The present study demonstrated that in the presence of T_3 , myocyte β -adrenergic responsiveness was significantly enhanced. In addition, in the presence of T_3 , cAMP production after β -adrenergic receptor stimulation was significantly augmented. However, T_3 alone did not significantly

increase cAMP production. Important conclusions from this portion of the study were twofold. First, the direct effects of T_3 on myocyte contractile function in the absence of β -adrenergic receptor stimulation appeared to be independent of cAMP-mediated events. Second, T_3 augmented cAMP production in response to β -adrenergic receptor stimulation within the myocyte. Therefore the findings of the present study provide a potential molecular mechanism for the past clinical observation of reduced inotropic requirements after T_3 administration.¹³

Several reports have suggested that T_3 may influence the myocyte sarcolemmal Na^+, K^+ -ATPase system.²⁶⁻²⁸ For example, Philipson and Edelman²⁶ demonstrated an increase in Na^+, K^+ -ATPase activity in myocardial homogenates after chronic treatment with T_3 in rats. Similar studies have suggested that Na^+, K^+ -ATPase activity and density increased after chronic exposure to T_3 .²⁸ The present study examined the immediate effects of T_3 on Na^+, K^+ -ATPase hydrolytic activity in the presence of T_3 . Thus the present study suggests that the immediate effects of T_3 on myocyte contractile performance were not mediated by direct effects on Na^+, K^+ -ATPase hydrolytic activity.

The L-type Ca^{+2} channel is an important sarcolemmal receptor system with respect to the modulation of Ca^{+2} entry into the myocyte and serves to trigger release of Ca^{+2} from the sarcoplasmic reticulum.³² Therefore the total amount of Ca^{+2} entering the myocyte through each L-type Ca^{+2} channel during channel opening periods is a fundamental determinant of the total amount of Ca^{+2} available to the contractile apparatus.³² The total amount of Ca^{+2} entering the myocyte through the L-type Ca^{+2} channel is directly proportional to the peak current and the duration that the L-type Ca^{+2} channel remains open.⁵³ In the present study, a significant shift was observed in the voltage at which peak L-type Ca^{+2} channel current occurred after T_3 administration. This immediate effect of T_3 on the L-type Ca^{+2} channel I-V relation may have caused increased Ca^{+2} entry through the channel with an augmentation of Ca^{+2} release from the sarcoplasmic reticulum and subsequent enhancement of myofilament crossbridging. Therefore one potential mechanism for the direct effects of T_3 on myocyte contractile performance may be modulation of the L-type Ca^{+2} channel opening periods. Future studies directed at more careful quantitation of L-type Ca^{+2} channel gating properties, specifically the probability of channel opening periods, would be

necessary to more carefully address this issue. β -Adrenergic receptor stimulation with subsequent cAMP production has been previously demonstrated to cause phosphorylation of the L-type Ca^{+2} channels and thereby increase peak L-type Ca^{+2} channel current.^{32,53} Consistent with this past observation, the present study demonstrated that β -adrenergic receptor stimulation significantly increased L-type Ca^{+2} channel current. More important, in the presence of both T_3 and β -adrenergic receptor stimulation, L-type Ca^{+2} channel current increased further from values with β -adrenergic receptor stimulation alone. Results from the present study demonstrated that T_3 augmented β -adrenergic receptor-stimulated cAMP generation. Accordingly, a potential mechanism for the augmentation in L-type Ca^{+2} channel current density in the presence of both T_3 and β -adrenergic receptor stimulation was probably increased phosphorylation of the L-type Ca^{+2} channel itself.

A final result from the activation of the β -adrenergic receptor system or increased L-type Ca^{+2} channel current is an elevation in myocyte intracellular Ca^{+2} content. In the present study intracellular Ca^{+2} levels were determined in isolated myocytes in real time with use of the Ca^{+2} -specific dye, fura 2/acetomethoxy ester.⁴⁴ Through this approach it could be determined whether T_3 alone directly modulated myocyte intracellular Ca^{+2} levels. Results from the present study demonstrated that no significant change in intracellular Ca^{+2} levels occurred after the addition of T_3 . These findings are consistent with those of a past report by Wicomb and associates⁵⁴ that demonstrated that no change in intracellular Ca^{+2} concentrations occurred in the presence of T_3 . One potential reason for the increase in myocyte contractile function after the addition of T_3 in this isolated myocyte model may be that the standard culture medium does not contain T_3 . Thus the increase in myocyte contractile function after the addition of T_3 may be a result of the normalization of T_3 concentrations found in plasma. In the present study, myocyte peak intracellular Ca^{+2} levels were significantly increased after β -adrenergic receptor stimulation. This finding is consistent with the downstream effects of β -adrenergic receptor stimulation.^{29,30} More important, in the presence of both T_3 and β -adrenergic receptor stimulation, myocyte intracellular Ca^{+2} levels increased significantly higher than those values obtained with β -adrenergic receptor stimulation alone. These findings demonstrated for the first time that

T_3 has the capacity to directly regulate intracellular Ca^{+2} availability to the contractile apparatus after β -adrenergic receptor stimulation. Therefore a contributory molecular mechanism for the improved myocyte β -adrenergic responsiveness in the presence of T_3 is increased myocyte intracellular Ca^{+2} availability.

Fundamental approaches for improving LV pump function after cardiopulmonary bypass and in the critical care setting require manipulation of the β -adrenergic receptor transduction system. In present clinical practice, the therapeutic approach to increasing LV contractile state is through the use of β -adrenergic receptor agonists or phosphodiesterase inhibitors, both of which result in increased cAMP content within the myocyte. However, long-term β -adrenergic receptor stimulation is associated with a significant number of complications, which include receptor desensitization and downregulation, arrhythmias, and hemodynamic instability.^{55,56} Therefore a continuing area of research is the pursuit of unique and alternative pharmacologic therapy for improving LV contractile performance. The present study demonstrated that T_3 improved myocyte contractile performance and was associated with modulation of the L-type Ca^{+2} channel. Furthermore, T_3 augmented myocyte β -adrenergic receptor transduction and subsequent Ca^{+2} availability to the contractile apparatus. Therefore the present study suggests that T_3 may provide a unique therapeutic modality, particularly as an adjunct to conventional β -adrenergic receptor agonist therapy.

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Discussion

Dr. Dimitri Novitzky (Tampa, Fla.). It is an honor to discuss this manuscript because I have been involved since 1983 in initial developments for the clinical indications of the use of triiodothyronine for nonthyroid conditions. The rapid reduction of plasma free T_3 has been observed in acute stress states with concomitant elevated plasma catecholamine levels. The tissue T_3 synthesis is markedly impaired and the mono-deiodination of thyroxine results in the production of reverse T_3 , a metabolically inactive compound. Experimental data have shown depletion of intracellular T_3 that is associated with abnormalities in the calcium metabolism, down regulation of beta receptors, and uncoupling of adenylate cyclase. As patients require higher doses of exogenous catecholamines, tachyphylaxis complicates the clinical picture, initiating the downward spiral course of multiorgan failure and death.

This has been observed in several clinical conditions such as in brain death, in heart operations with cardiopulmonary bypass, in myocardial infarction, in severe cardiac failure, and in all shock states.

The data obtained after the induction of experimental brain death in animals show that there is a rapid depletion of plasma free T_3 and it is associated with a catecholamine storm, thus resulting in diffuse structural myocardial injury and depletion of high-energy phosphates and lactate accumulation. Replacement of triiodothyronine in brain-dead animals rapidly restored the aerobic metabolism, correcting the tissue lactic acidosis and high-energy phosphate levels. Rapid recovery of myocardial dysfunction was observed in an ex vivo model no different from that of hearts procured from live animals. Today, T_3 has become a therapeutic modality in organ transplantation.

We administered triiodothyronine for the first time in 1986 to a patient dependent on cardiopulmonary bypass used for emergency valve replacement because of bacterial endocarditis and sepsis. The patient was supported by cardiopulmonary bypass, dependent on high-dose inotropic support, and remained in ventricular fibrillation despite all therapeutic interventions. After T_3 administration, cardiac recovery was observed within minutes, the heart converted to sinus rhythm, and the inotropic support was rapidly reduced. The patient was later discharged home.

This clinical observation led us to our extensive experimental work in animals, in which we observed recovery of these rapid high-energy phosphates and prevention of tissue lactate in baboons.

We have currently administered T_3 to patients with high-risk conditions, and in our experience of 134 patients

with a mean estimated mortality rate of 29% the mortality has been reduced by 73%.

The authors have clearly shown that the rapid extranuclear effects of T_3 bypass the deoxyribonucleic acid-ribonucleic acid-protein synthesis and have observed an increment of the calcium availability to the sarcomeres and the synergistic effects with beta receptors enhancing the contractile process in the isolated myocyte. Obviously further studies are required to elucidate the interaction of the myocyte, the endothelium, the autonomic system, and the whole components of blood, not only within the heart but also at the systemic level and as concerns other organs.

My first question is this: have the authors looked at the prolonged effects of adrenergic stimulation of the isolated myocyte? Has tachyphylaxis been observed and have the authors tried to reverse this by T_3 ?

Second, because T_3 works via calcium fluxes and there is rapid re compartmentalization of free ionized calcium mainly within the sarcoplasmic reticulum, would the authors speculate that pretreatment with T_3 before an ischemic event might prevent or reduce the reperfusion injury such as observed in the stunned myocardium?

Finally, because the beneficial effect of T_3 on the cardiovascular system directly affects the contractile state of the heart and reduces the systemic vascular resistance, would the authors consider expanding their study to look at isolated myocytes procured from peripheral smooth muscle arterioles and the interaction of T_3 with vasoactive agents?

I would like to congratulate the authors once more for

this excellent work in which molecular biology and clinical observations go hand in hand.

Dr. Walker. In this model of isolated myocytes we have looked at the effects of T_3 independent of neurohormonal influences and independent of loading conditions. We have actually examined the changes in calcium levels, but we have not examined fluxes in calcium and whether these are affected by ischemia. The model that we use to examine myocytes that is more clinically relevant to patients with dilated cardiomyopathy is not an ischemic model, it is a model of dilated cardiomyopathy in which there is no ischemia. Therefore we have not looked at those problems.

Finally, we have not looked at the effects of T_3 on loading conditions specifically because that has been done by other investigators. The purpose of these studies was to see whether T_3 had its effects directly at the level of the myocytes.

Dr. Andrew S. Wechsler (Richmond, Va.). How do the authors explain that in these isolated cell suspensions they have always seen a positive inotropic response? This differs from results of most of the studies in whole organs in which it has been difficult to demonstrate a positive inotropic response except after ischemia.

Dr. Walker. The positive response to T_3 in the isolated myocytes used in our studies was measured in an environment devoid of thyroid hormone. This mimics the low T_3 state seen with ischemia where other investigators have seen a positive inotropic response in whole organs.

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