Modulation by the Thyroid State of Intracellular Calcium and Contractility in Ferret Ventricular Muscle

Roderick MacKinnon, Judith K. Gwathmey, Paul D. Allen, G. Maurice Briggs, and James P. Morgan

The purpose of this study was to determine whether the cardiac contractile abnormalities induced by hyperthyroidism or hypothyroidism are caused by changes in intracellular Ca\(^{2+}\) handling or by alterations in the distribution of isoenzymes of ventricular myosin. Right ventricular papillary muscles obtained from euthyroid ferrets and ferrets treated with l-thyroxine (hyperthyroid) or methimazole (hypothyroid) were loaded with the calcium indicator aequorin for recording intracellular Ca\(^{2+}\) levels during isometric contraction. In muscles from the hypothyroid ferrets, peak tension was reduced and the duration of contraction prolonged compared to the controls; these changes were associated with a Ca\(^{2+}\) transient of decreased amplitude and prolonged duration. Hyperthyroidism produced opposite changes in the time course of the Ca\(^{2+}\) transient and the associated isometric contraction. Native polyacrylamide gel electrophoresis was performed on myosin extracted from the right ventricular free wall of control and treated ferrets. The hyperthyroid state was associated with new myosin formation as indicated by the appearance of three myosin bands on the pyrophosphate gel. Gels of myosin from hypothyroid and euthyroid ferrets showed a single band that migrated with the slowest of the three bands from the hyperthyroid ferrets. These results suggest that changes in both Ca\(^{2+}\) handling and myosin isoenzymes may contribute to the contractile abnormalities observed in hyperthyroidism. Alterations in intracellular Ca\(^{2+}\) handling alone may account for the contractile changes induced by hypothyroidism. (Circulation Research 1988;63:1080-1089)

Thyroid hormone influences the contractile state of cardiac muscle by altering the rates of contraction and relaxation. However, it has been difficult to identify the steps in excitation-contraction coupling that are modified by changes in the thyroid state. In some species, thyroid hormone influences the distribution of myosin isoenzymes in ventricular muscle. In rabbits, the maximum velocity of papillary muscle shortening directly correlates with the fraction of thyroid hormone-induced V\(_1\) isozyme. The control of the distribution of ventricular myosin types represents a straightforward mechanism by which thyroid hormone can influence the mechanical properties of ventricular muscle. However, multiple ventricular myosins have been demonstrated in only a few species.

Evidence for another type of control, the thyroid-hormone-induced modulation of calcium handling by ventricular cells, has been provided by studies of isolated sarcoplasmic reticulum. Sarcoplasmic reticulum isolated from the ventricular muscle of hyperthyroid animals exhibits increased rates of calcium sequestration and calcium-magnesium ATPase activity; in contrast, these rates are decreased in sarcoplasmic reticulum from hypothyroid animals. Furthermore, measurements of transsarcolemma calcium isotope fluxes in cultured chick ventricular cells have revealed that thyroid hormone increases the quantity and rates of exchange of a rapidly exchangeable pool of intracellular calcium. These data indicate that thyroid hormone may influence the contractile properties of ventricular muscle by altering intracellular calcium han-
Because the contraction of cardiac muscle is a rapid, transient event, to determine whether or not changes in calcium handling may account for changes in the contractile properties, it is necessary to measure the rapid calcium transient which occurs during the contraction. Such measurements are possible in intact ventricular muscle with the bioluminescent protein aequorin as an intracellular calcium indicator. In the present study we have investigated in ferret papillary muscles the influence of the thyroid state on the isometric contraction and the calcium transient. In addition, by the technique of pyrophosphate (native) polyacrylamide gel electrophoresis, we have characterized the influence of the thyroid state on ferret ventricular myosin.

**Materials and Methods**

**Animal Preparations**

L-Thyroxine, 0.3 mg/kg, was injected subcutaneously daily for 2–3 weeks to induce the hyperthyroid state in 16 twelve-week-old ferrets. The sodium salt of L-thyroxine was dissolved in a 1:1 solution of 95% ethanol: 0.01N NaOH and diluted 4:1 with 0.9% NaCl at the time of injection. The euthyroid group included 11 age-matched ferrets that were not injected and four that were injected daily with vehicle. To induce the hypothyroid state, 0.2 g/l of methimazole was added to the drinking water of 14 ferrets for approximately 6 weeks beginning at the age of 8 weeks.

**Tension and Aequorin Signal Measurements**

Ferrets were anesthetized with chloroform. The hearts were excised, and right ventricular papillary muscles of 1.0 mm or less in diameter were selected for study. The muscle lengths (mm) and cross-sectional areas (mm²) used in these experiments were, respectively, euthyroid (n=16) 4.5±1.6, 0.71±0.88; hyperthyroid (n=17) 5.0±1.1, 0.92±0.34; and hypothyroid (n=14) 4.0±1.5, 0.88±0.26. After removal from the heart, the base of each papillary muscle was fixed to a muscle holder and the tendinous end to a Statham force transducer (Gould Instruments, Cleveland, Ohio). The bath contained a physiological saline solution of the following composition (mM): NaCl 120, KCl 5.9, dextrose 11.5, NaHCO₃ 25, NaH₂PO₄ 1.2, MgCl₂ 1.2, and CaCl₂ 2.5. The solution was bubbled with 95% O₂-5% CO₂ and equilibrated to pH 7.4 at 30°C. Lower than normal body temperature was used in these experiments because we have observed that ferret papillary muscle maintains a stable level of tension development for a longer period and has a lower incidence of dysrhythmic activity at 30°C compared with 38°C. To prevent precipitation of calcium, PO₄³⁻ was deleted during experiments in which the Ca²⁺ was varied. The muscle was stimulated with a square-wave pulse of 5 msec duration and threshold +10% voltage delivered through a punctate platinum electrode located at the base of the muscle. To further minimize the effects of catecholamine release from the adrenergic nerve endings, propranolol, 6×10⁻⁷ M, was added to the bath in a subset of five muscles from each thyroid group. The muscle was stretched to the length at which maximum isometric tension developed.

Aequorin was loaded by a chemical procedure that involves three steps. In step one, the muscle was exposed to a Ca²⁺-free solution; in step two, it was exposed to aequorin, and in the third step, Ca²⁺ was slowly added back to the solution. After aequorin loading, the muscles were returned to the physiological saline solution and stimulated to contract at 0.33 Hz at 30°C. A light collecting apparatus designed by Dr. J.R. Blinks was used to simultaneously measure the aequorin signal and isometric tension. The light signal was passed through a preamplifier with a filter time constant of 5 msec. Both light and tension signals were recorded with an FM tape recorder. To improve the signal-to-noise ratio, between 15 and 300 signals were averaged while the muscle contracted at steady state.

In some of the muscles, the aequorin signal was converted to a Ca²⁺ concentration. In some experiments, after exposure of the muscles to high Ca²⁺ concentrations or certain drugs, we noticed an increase in resting luminescence that may have been caused by cell injury. Because of this, we did not quantitate Ca²⁺ in every experiment by the method of fractional luminescence (see below). Instead, the calibration procedure only was performed immediately after aequorin loading in muscles from eight animals in each thyroid group. These muscles were cooled to 22°C and stimulated to contract. After a steady state was reached, tension and the light signal were recorded. In each group, five of eight determinations were performed in the presence of propranolol, 6×10⁻⁷ M. Lmax, the aequorin signal that would be recorded if all of the aequorin in the preparation were instantly exposed to a saturating Ca²⁺ concentration, was calculated by the method of fractional luminescence. Back-ground light (L) at 22°C was determined with the shutter open and the unloaded muscle in the light collecting apparatus; if significant, this value per unit of time was subtracted from the integral of Lmax. In most experiments background L was not distinguishable from the dark current of the tube. At 22°C the muscle was exposed to the same physiological salt solution, minus PO₄³⁻, with 20 mM CaCl₂ and 1% Triton X-100. It generally required several hours for all of the aequorin to be consumed. During lysis, the light signal was recorded on chart paper. Stability of the baseline throughout lysis was checked by repeatedly closing, for brief periods, the shutter between the muscle and the photomultiplier tube to evaluate amplifier drift. By this method we found that drift was negligible compared with the amplitude of the aequorin signal. The time integral of luminescence was determined manually. The product of the time integral of the aequorin signal
during detergent lysis and the rate constant for aequorin consumption in saturating \( \text{Ca}^{2+} \), 1.2 mM Mg\(^{2+} \) (preincubated) at 22\(^\circ\) C (0.86 sec\(^{-1}\) was taken as \( L_{max} \). \( L/L_{max} \) is the ratio of the aequorin signal over \( L_{max} \) for the muscle, was then transformed into \( \text{Ca}^{2+} \) concentrations with an in vitro calibration curve. The calibration curve was generated by measuring \( L/L_{max} \) at 22\(^\circ\) C for an aliquot of aequorin added to a salt solution of known \( \text{Ca}^{2+} \) concentration. The salt solution contained KCl 150 mM, PIPES 5 mM, MgCl\(_2\) 1.2 mM, pH 7.0, EGTA, and \( \text{Ca}^{2+} \) were added to set the free \( \text{Ca}^{2+} \) concentration. The aequorin was pre-incubated in the same solution minus \( \text{CaCl}_2 \).

**Myosin Studies**

Immediately after removal of the heart from the ferret, a small portion of the right ventricular free wall was isolated, weighed and then frozen at -70\(^\circ\) C for further study. Care was taken to avoid atrial contamination. Myosin was extracted from approximately 2 to 10 mg of the frozen tissue. The tissue was first washed for 3–4 hours in 10 volumes of washing buffer containing 50% glycerol, 10 mM Tris HCl, 1 mM EGTA, 0.1 mM dithiothreitol, pH 7.5 at 4\(^\circ\) C, to remove some of the soluble protein. Then the myosin was extracted overnight into a buffer containing 50% glycerol, 50 mM Na\(_2\)P\(_2\)O\(_7\), 1% \( \beta \)-mercaptoethanol (pH 8.8 at 4\(^\circ\) C). Myosin was examined by both one-dimensional sodium dodecyl sulfate (SDS) and pyrophosphate (native) polyacrylamide gel electrophoresis to examine the myosin purity (SDS) and to determine the number of isoenzymatic forms (pyrophosphate). SDS gels were run according to the method of Laemmli, with a 4.5% stacking and a 13% running gel.

Pyrophosphate (native) gel electrophoresis was performed by the method of Hoh. These 3% acrylamide gels were run at 2–4\(^\circ\) C for 16 hours, with recirculation of the buffer between the anodal and cathodal reservoirs.

**Analysis**

Results are expressed as mean±SD. Control and test results were compared by a Student's \( t \) test. In cases where aequorin signals were transformed to absolute \( \text{Ca}^{2+} \) concentrations, the signals were converted from analog to digital display at a frequency of 1 kHz. The transformations were made with a Digital Equipment Corporation computer (LSI 11/23, Marlboro, Massachusetts).

**Chemicals**

L-Thyroxine, (-)-propranolol, and methimazole were obtained from Sigma Chemical, St. Louis, Missouri. EGTA was obtained from Baker, Phillipsburg, New Jersey, and Triton X-100 was from Fisher Scientific, Fair Lawn, New Jersey. Acrylamide and methylene bis-acrylamide were obtained from Biorad, Richmond, California. The aequorin used in these studies was purchased from the laboratory of J.R. Blinks in Rochester, Minnesota.

**Results**

The influence of the thyroid state on ferret body weight and heart weight is shown in Table 1. Both the hyperthyroid and hypothyroid ferrets weighed less than the euthyroid controls. The very low body weight of the hyperthyroid ferrets may reflect the fact that the hyperthyroid state was induced over a 6 week period beginning at the age of 8 weeks, whereas the hyperthyroid state was induced over approximately 2 weeks before study. Although the hyperthyroid ferrets weighed less than the controls, their hearts were larger. This is consistent with cardiac hypertrophy that is known to occur as a result of the hyperthyroid state. In both the absence and presence of \( \beta \)-adrenergic blockade with propranolol, the thyroid state had a profound influence on the isometric contraction (Table 2). In the hyperthyroid ferrets the peak isometric tension (T) was not different from that in euthyroid ferrets. Muscles from hypothyroid ferrets, however, generated significantly less tension. The large standard deviation associated with the value T for the hypothyroid group in the absence of propranolol is reflective of a single muscle which generated 22.4 mN/mm\(^2\). The thyroid state exerted a striking effect on the duration of isometric contraction. Both the time from stimulus to peak of isometric contraction (TPT), and the time from the peak to an 80% relaxation (\( T_{80} \)), were prolonged in muscles from hypothyroid ferrets and abbreviated in muscles from hyperthyroid ferrets as compared with the corresponding values in euthyroid ferrets. These time course changes are consistent with the results of previous studies on the influence of the thyroid state on the mechanical properties of cardiac muscle.
peak of the aequorin signal (TPL) and the time from the peak to an 80% decline (L_{0.8}), were measured in muscles from hypothyroid, euthyroid, and hyperthyroid ferrets. The results are summarized in Table 3. The response of the aequorin signal to rapid changes in Ca^{2+} concentrations, such as occurs during the rise phase of the transient, results in filtering of the rapid concentration change.\(^1\)\(^7\)\(^8\) Despite this limitation, on average, TPL in the hyperthyroid ferrets was briefer than in euthyroid ferrets; this trend did not achieve statistical significance in the smaller number of animals studied after propranolol treatment. Although TPL is a function of both the rise phase and the decay phase of the Ca^{2+} transient, on average, TPL in the hyperthyroid ferrets was longer than in the euthyroid ferrets. The peak of isometric contraction (T) in milliNewtons per square millimeter is listed for the three thyroid states. Measures of the duration of the peak isometric contraction include the time to peak tension (TPT) and the time from the peak to 80% relaxation (T_{0.8}). The values obtained for T, TPT and T_{0.8} are not significantly different in the 0 vs 6×10^{-7} M propranolol groups. T is not different in the control versus the hyperthyroid ferrets of both the 0 and 6×10^{-7} M propranolol groups. All other comparable pairs are significantly different as indicated by asterisks: *p=0.03, **p=0.01, ***p=0.001. Stimulation frequency, 0.33 Hz. Temperature, 30°C.

### Table 2. Influence of the Thyroid State on the Isometric Contraction

<table>
<thead>
<tr>
<th></th>
<th>Hypothyroid</th>
<th>Euthyroid</th>
<th>Hyperthyroid</th>
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<tbody>
<tr>
<td>No propranolol in bath</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>T (mN/mm²)</td>
<td>6.2±6.6**</td>
<td>14.2±6.7</td>
<td>15.3±7.3</td>
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<tr>
<td>TPT (msec)</td>
<td>228±24**</td>
<td>200±19</td>
<td>152±17***</td>
</tr>
<tr>
<td>T_{0.8} (msec)</td>
<td>200±20***</td>
<td>167±19</td>
<td>124±15***</td>
</tr>
<tr>
<td>Propranolol, 6×10^{-7} M in bath</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>T (mN/mm²)</td>
<td>3.3±1.5**</td>
<td>12.7±6.4</td>
<td>13.9±8.1</td>
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<tr>
<td>TPT (msec)</td>
<td>250±42**</td>
<td>185±19</td>
<td>151±14**</td>
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<td>T_{0.8} (msec)</td>
<td>205±46*</td>
<td>143±17</td>
<td>118±11*</td>
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</tbody>
</table>

The peak of isometric contraction (T) in milliNewtons per square millimeter is listed for the three thyroid states. Measures of the duration of the peak isometric contraction include the time to peak tension (TPT) and the time from the peak to 80% relaxation (T_{0.8}). The values obtained for T, TPT and T_{0.8} are not significantly different in the 0 vs 6×10^{-7} M propranolol groups. T is not different in the control versus the hyperthyroid ferrets of both the 0 and 6×10^{-7} M propranolol groups. All other comparable pairs are significantly different as indicated by asterisks: *p=0.03, **p=0.01, ***p=0.001. Stimulation frequency, 0.33 Hz. Temperature, 30°C.

### Table 3. Influence of the Thyroid State on the Time Course of the Ca^{2+} Transient

<table>
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<th>Hypothyroid</th>
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<tr>
<td>n</td>
<td>9</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>TPL (msec)</td>
<td>57±7</td>
<td>55±6</td>
<td>49±6*</td>
</tr>
<tr>
<td>L_{0.8} (msec)</td>
<td>119±21***</td>
<td>91±8</td>
<td>68±8***</td>
</tr>
<tr>
<td>Propranolol, 6×10^{-7} M in bath</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>TPL (msec)</td>
<td>71±9</td>
<td>62±11</td>
<td>51±9</td>
</tr>
<tr>
<td>L_{0.8} (msec)</td>
<td>109±18*</td>
<td>86±8</td>
<td>66±12**</td>
</tr>
</tbody>
</table>

The response of the aequorin signal to rapid changes in Ca^{2+} concentrations, such as occurs during the rise phase of the transient, results in filtering of the rapid concentration change.\(^1\)\(^7\)\(^8\) Despite this limitation, on average, TPL in the hyperthyroid ferrets was longer than in euthyroid ferrets; this trend did not achieve statistical significance in the smaller number of animals studied after propranolol treatment. Although TPL is a function of both the rise phase and the decay phase of the Ca^{2+} transient, on average, TPL in the hyperthyroid ferrets was longer than in the euthyroid ferrets. The peak of isometric contraction (T) in milliNewtons per square millimeter is listed for the three thyroid states. Measures of the duration of the peak isometric contraction include the time to peak tension (TPT) and the time from the peak to 80% relaxation (T_{0.8}). The values obtained for T, TPT and T_{0.8} are not significantly different in the 0 vs 6×10^{-7} M propranolol groups. T is not different in the control versus the hyperthyroid ferrets of both the 0 and 6×10^{-7} M propranolol groups. All other comparable pairs are significantly different as indicated by asterisks: *p=0.03, **p=0.01, ***p=0.001. Stimulation frequency, 0.33 Hz. Temperature, 30°C.
Figure 1. The thyroid state influences the time course of the isometric contraction and the Ca\(^{2+}\) transient. The isometric tension (T) and the aequorin light signal (L) were recorded from a hypothyroid (A), euthyroid (B), and hyperthyroid (C) ferret at 30° C; 0.33 Hz stimulation. T is expressed in milliNewtons per square millimeter of muscle cross-sectional area. Because the maximum aequorin luminescence was not measured in these preparations, the scale is arbitrary. The Ca\(^{2+}\) transients are scaled to equal amplitudes and superimposed in D. Note that in D, the time scale is different. In E, the tensions have been scaled to equal amplitudes and superimposed. The time from the beginning of the stimulus sweep (S) to the stimulus represents 100 milliseconds.

are not a manifestation of different Ca\(^{2+}\) levels, but more likely reflect a difference in the rate of cytoplasmic Ca\(^{2+}\) removal at any given intracellular Ca\(^{2+}\) concentration.

Although the differences in the time course of the Ca\(^{2+}\) transient are not the result of different intracellular Ca\(^{2+}\) levels per se, it is appropriate to ask whether or not the thyroid state influences the levels of Ca\(^{2+}\). In order to answer this question L\(_{\text{max}}\) was estimated, as described in "Materials and Methods," in muscles from eight animals from each of the thyroid groups. Propranolol, 6×10\(^{-7}\) M, was present in five of the experiments from each group. By transforming the aequorin signal into Ca\(^{2+}\) concentrations, an estimate of the intracellular Ca\(^{2+}\) levels can be obtained, as well as an appreciation for the nonlinearity of the aequorin signal. Because of the possibility of there being an unknown fraction of injured cells (see "Materials and Methods") the measurement of resting Ca\(^{2+}\) must be regarded as an upper limit estimate of the true physiological Ca\(^{2+}\) concentration. Moreover, although damaged cells would increase the resting glow, they might reduce the amplitude of the apparent Ca\(^{2+}\) transient if the cells were uncoupled, but still contained active aequorin that could contribute to the estimate of L\(_{\text{max}}\). Even in the absence of cell injury, the transformation of L/L\(_{\text{max}}\) to Ca\(^{2+}\) is associated with uncertainties which prohibit the establishment of accurate absolute Ca\(^{2+}\) levels (see "Discussion"). Because of these limitations, estimates of absolute Ca\(^{2+}\) were made only to compare relative differences in calcium levels between muscles. The aequorin signal and associated tension, measured in a papillary muscle from a hypothyroid animal at 22° C, is illustrated in Figure 4A. The aequorin signal has been transformed into a Ca\(^{2+}\) concentration at each point in time and the results are illustrated in Figure 4B. The calibration curve is shown in Figure 4C. Note that the Ca\(^{2+}\) level in Figure 4B returns to baseline only shortly before tension returns to baseline. This is difficult to appreciate when viewing the non-transformed signal.

Table 4 summarizes the peak and resting intracellular Ca\(^{2+}\) concentrations measured at 22° C in papillary muscles from hypothyroid, euthyroid, and hyperthyroid ferrets. The resting Ca\(^{2+}\) levels were not different in the three thyroid states. Because of the nonlinearity of the aequorin signal, even a very small number of injured cells with high resting Ca\(^{2+}\) concentrations could introduce a large error into the estimate of the average resting Ca\(^{2+}\). However, at the peak of the transient, all of the cells in the preparation have a high Ca\(^{2+}\) concentration. Therefore, a small number of injured cells will contribute much less significantly to the overall signal and result in a less significant error. At the peak of the transient the mean Ca\(^{2+}\) level in the hypothyroid
ferrets was lower than in euthyroid ferrets. The $Ca^{2+}$ concentration measured at the peak of the $Ca^{2+}$ transient in hyperthyroid ferrets was not different from that in euthyroid ferrets.

Because the thyroid state is known to modulate the distribution of ventricular myosin in some animals,\textsuperscript{2,19} and because the influence of the thyroid state on ferret ventricular myosin has not been reported, pyrophosphate (native) and SDS gel electrophoresis were performed on myosin extracted from the right ventricular free walls of several of the hypothyroid, euthyroid, and hyperthyroid ferrets. On the SDS gel, there are no differences between myosin from hypothyroid, euthyroid, and hyperthyroid ferrets (data not shown). Figure 5 shows the results of pyrophosphate gel electrophoresis from a ferret in each of the thyroid groups. In all of the hyperthyroid animals studied ($n=7$), new, more rapidly migrating myosin was present. In all of the hypothyroid ($n=10$) and euthyroid ($n=5$) ferrets, there was a single band that migrated with the slowest migration band of the hyperthyroid ferrets.

For present purposes, the three bands have been termed $V_3$, $V_2$, and $V_1$. Comigration studies of normal ferret and rat ventricular isomyosins demonstrate that the single band in ferret comigrates with $V_1$ of the rat. However, the relation between the two additional isozymes in ferrets and the $V_3$, $V_2$, and $V_1$ myosins of the rat and rabbit is yet to be determined. In the ferret, the separation between $V_3$ and $V_1$, or the top two bands in Figure 5C, was not easy to distinguish in all animals. Good separation of the three isozymes was possible only with low protein loading. Although further characterization of these myosins is necessary, at this point it is clear that by polyacrylamide gel electrophoresis, the hyperthyroid state is associated with new ventricular myosin formation, whereas the myosin in the hypothyroid state appears to be identical to that in the control state.

**Discussion**

As we have previously reported for the hyperthyroid state alone,\textsuperscript{20,21} the present study demonstrates that both hypothyroidism and hyperthyroidism influence the $Ca^{2+}$ transient in ventricular muscle during excitation-contraction coupling. Aequorin is a very good indicator for monitoring $Ca^{2+}$ transients in muscle since it is easier to avoid problems with motion artifacts in aequorin signals than in signals obtained with most other $Ca^{2+}$ indicators.\textsuperscript{22} One limitation associated with the use of aequorin, however, is its rather slow response to a rapid change in $Ca^{2+}$ concentration. At 21°C aequorin responds with a time constant of around 10 msec.\textsuperscript{17,18} As a consequence, there is significant filtering of the rapid $Ca^{2+}$ concentration change that occurs during the rise phase of the $Ca^{2+}$ transient. Despite this limitation, on average TPL was abbreviated and the rate of rise was faster in muscle from hyperthyroid ferrets. The same trend was observed in the presence of $\beta$-adrenergic blockade with propranolol. The results of studies with drugs that are known to inhibit sarcoplasmic reticular function indicate that the rise phase of the $Ca^{2+}$ transient appears to reflect the release of $Ca^{2+}$ from the sarcoplasmic reticulum.\textsuperscript{23} The faster rate of rise of the $Ca^{2+}$ transient in the hyperthyroid state may reflect an enhancement of the processes that initiate the release of $Ca^{2+}$ from the sarcoplasmic reticulum. Alternatively, an increase in either the quantity of the sarcoplasmic reticular membrane, the transmembrane $Ca^{2+}$ gradient or the membrane $Ca^{2+}$ conductance would lead to a faster rate of rise. Although these latter mechanisms might be expected to result in a greater quantity of $Ca^{2+}$ at the peak of the $Ca^{2+}$ transient, the faster rate of removal of free myoplasmic $Ca^{2+}$ observed in muscles from hyperthyroid ferrets would be expected to lower the $Ca^{2+}$ at the peak of the transient.

The thyroid state exerts its most striking and consistent effect on the decay phase of the $Ca^{2+}$ transient. The decay phase reflects the removal of free myoplasmic $Ca^{2+}$ following the sudden increase initiated by an action potential. There are no doubt several processes which account for the rapid restoration back to resting levels of myoplasmic $Ca^{2+}$, including the buffering of $Ca^{2+}$ by intracellular binding sites, the extrusion of $Ca^{2+}$ from the cell by sarcolemmal mechanisms and the sequestration of $Ca^{2+}$ by the sarcolemmal membrane. The relative importance in cardiac muscle of each of these mechanisms is not known with certainty. Estimates of the $Ca^{2+}$ binding capacity of cardiac sarcoplasmic

\[ \text{FIGURE 2. Correlation between time course of isometric relaxation and the decay of the Ca}^{2+} \text{ transient. L}_\text{s} \text{ and } T_\text{s}, \text{ as defined in the text, are plotted against each other. Both axes are in milliseconds. Stimulation frequency 0.33 Hz. Temperature 30° C.} \]
The time courses of isometric contraction and the Ca\(^{2+}\) transient are independent of signal amplitude. The isometric tension (T) is in milliNewtons per square millimeter and the aequorin signal (L) is in nanoamperes. The interval from the beginning of the stimulus sweep to the stimulus artifact is 100 milliseconds. In A, B, and C the muscle contracted at steady state in the Ca\(^{2+}\) concentrations listed. In D and E, the aequorin signals and tension, respectively from A, B, and C, were scaled to equal amplitudes and superimposed. Stimulation frequency 0.33 Hz. Temperature, 30° C.

reticulum indicate that this organelle is capable of binding enough Ca\(^{2+}\) to account for cardiac muscle relaxation. The present study shows that, in muscles from hypothyroid ferrets, the rate of Ca\(^{2+}\) removal was significantly slower, while in muscles from hyperthyroid ferrets the rate of Ca\(^{2+}\) removal was significantly faster, than in muscles from euthyroid ferrets. These results are consistent with studies that have demonstrated a decreased rate of Ca\(^{2+}\) sequestration by cardiac muscle sarcoplasmic reticulum isolated from hypothyroid animals versus an increased rate by sarcoplasmic reticulum isolated from hyperthyroid animals. It is also interesting to correlate these results with the demonstration in the rat that the hyperthyroid state results in hypertrophy of the sarcoplasmic reticulum of slow twitch skeletal muscle and the hypothyroid state results in a decrease in the total quantity of sarcoplasmic reticulum of fast twitch skeletal muscle. Finally, it is possible that the thyroid state might also influence the kinetics of Ca\(^{2+}\) binding and/or the number of intracellular Ca\(^{2+}\) binding sites. In particular,

Conversion of the aequorin signal to Ca\(^{2+}\) concentration. Panel A shows the relation between the aequorin signal and isometric tension during contraction of a papillary muscle from a hypothyroid ferret. Stimulation frequency 0.33 Hz, 22° C. In panel B, the aequorin signal has been transformed into Ca\(^{2+}\) concentrations as described in the methods. The transformation was performed using the calibration curve shown in panel C. The calibration was performed under the following conditions: KCl 150 mM, PIPES, 5mM, MgCl 1.2 mM, pH 7.0, 22° C. All measurements were made at 22° C.
since troponin C is the largest Ca\(^{2+}\) sink in the cell, a change in its Ca\(^{2+}\)-affinity would be expected to have a major effect on the amplitude and time course of the calcium transient. However, as discussed below, evidence has accumulated which suggests that changes in the thyroid state do not affect the Ca\(^{2+}\) affinity of troponin C.27

The conversion of the aequorin signal to absolute Ca\(^{2+}\) levels is associated with several uncertainties. One of these stems from the fact that the sensitivity of aequorin for Ca\(^{2+}\) is a function of the Mg\(^{2+}\) concentration.22 The concentration of this ion in ferret ventricular muscle is not known with certainty (but see Blatter and McGuigan28). A second uncertainty arises from the fact that under defined conditions, a given L/L\(_{\text{max}}\) is associated with a single concentration of Ca\(^{2+}\) only when the distribution of Ca\(^{2+}\) in the field of study is uniform.22 Because intracellular Ca\(^{2+}\) gradients no doubt exist at least at the peak of the transient, the aequorin signal will yield an over-estimation of the true average Ca\(^{2+}\) concentration. These considerations are relevant to our finding that the amplitude of the aequorin signal recorded in muscles from the hypothyroid animals are lower than in the controls. One interpretation of this finding is that the same quantity of Ca\(^{2+}\) is released into the cytoplasm of muscles from hypothyroid and euthyroid ferrets, but that Ca\(^{2+}\) gradients at the peak of the transient are lower in the former group. At present, using aequorin, we

\[
\begin{array}{ccc}
\text{Hypothyroid} & \text{Euthyroid} & \text{Hyperthyroid} \\
(n=8) & (n=8) & (n=8) \\
\text{Resting} & & \\
\log L/L_{\text{max}} & -5.8\pm0.3 & -5.7\pm0.2 & -5.9\pm0.3 \\
\text{Ca}^{2+} (\text{M}) & (2.9\pm1.4)\times10^{-7} & (3.0\pm0.6)\times10^{-7} & (2.5\pm1.4)\times10^{-7} \\
\text{Peak} & & \\
\log L/L_{\text{max}} & -4.2\pm0.6^* & -3.5\pm0.3 & -3.6\pm0.3 \\
\text{Ca}^{2+} (\text{M}) & (1.7\pm0.9)\times10^{-4} & (2.9\pm0.8)\times10^{-4} & (2.6\pm0.9)\times10^{-4} \\
\end{array}
\]

L/L\(_{\text{max}}\) and Ca\(^{2+}\) concentration during rest and at the peak of the Ca\(^{2+}\) transient were measured as described in "Materials and Methods." The measurements were made at 22° C. Each value represents the mean±SD; asterisks indicate significant difference from control: *p≤0.01, **p≤0.001.

\[\text{FIGURE 5. Pyrophosphate gel electrophoresis of ventricular myosin from control and treated ferrets. Pyrophosphate gel electrophore-to-grams of ventricular myosin from hypothyroid (A), euthyroid (B), and hyperthyroid (C) ferrets.}\]
are unable to distinguish this possibility from the interpretation that there is less free myoplasmic Ca\(^{2+}\) at the peak of the transient in muscles from hypothyroid animals. If there is less Ca\(^{2+}\), this may at least partially account for the fact that muscles from hypothyroid animals develop reduced peak isometric tension. This interpretation is also consistent with the demonstration in cultured chick ventricular myocytes that treatment with thyroid hormone results in a larger rapidly exchangeable Ca\(^{2+}\) pool.\(^6\)

Having established the fact that intracellular Ca\(^{2+}\) handling is significantly influenced by the thyroid state, what can we say about the relation between the changes in Ca\(^{2+}\) handling and the observed mechanical properties? Certainly the hypothyroid state influences the Ca\(^{2+}\) transient in a way that favors a prolongation of the duration of contraction, while changes in the hyperthyroid state favor an abbreviated contraction (see Figure 2). To what extent, though, does the altered Ca\(^{2+}\) handling account for the altered mechanical properties? The hyperthyroid state in the ferret probably exerts some of its influence on contraction by inducing the formation of new ventricular myosin. Therefore, there appear to be at least two mechanisms by which excessive thyroid hormone may alter the contractility of ventricular muscle in the ferret. On the other hand, the situation appears to be simpler in the hypothyroid states in ferrets. Compared with euthyroid ferrets there is no alteration in the distribution of ventricular myosins; we have identified only a change in Ca\(^{2+}\) handling. Furthermore, it has been demonstrated in skinned rabbit ventricular muscle that the thyroid state does not influence the steady state force versus [Ca\(^{2+}\)] relation.\(^27\) This is strong evidence that at least in rabbits, the Ca\(^{2+}\) affinity of the binding sites which control force generation is not altered by the thyroid state. However, it would be premature at this point to conclude that Ca\(^{2+}\) handling alone underlies the hypothyroid-induced mechanical changes, because when a muscle contracts it is not under steady state conditions. The thyroid state could theoretically alter a step between the binding of Ca\(^{2+}\) to troponin C and the generation of force in a way that would change the relation between the rate of change of the Ca\(^{2+}\) concentration and the rate of change of force. Despite the remaining uncertainties, this study presents convincing evidence that the modulation of intracellular Ca\(^{2+}\) handling is an important mechanism by which the thyroid state alters the contractile properties of ventricular muscle.

Acknowledgments

The authors gratefully acknowledge the contributions of Nancy Hague and Cynthia Perrault, and thank George Moody for his assistance with computer programming and analysis.

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**KEY WORDS**  • calcium indicators • hyperthyroidism • hypothyroidism • aequorin • hypertrophy
Modulation by the thyroid state of intracellular calcium and contractility in ferret ventricular muscle.
R MacKinnon, J K Gwathmey, P D Allen, G M Briggs and J P Morgan

Circ Res. 1988;63:1080-1089
doi: 10.1161/01.RES.63.6.1080

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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