Selective Binding of L-Thyroxine by Myosin Light Chain Kinase*

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L-Thyroxine selectively inhibited Ca\textsuperscript{2+}-calmodulin-activated myosin light chain kinases (MLC kinase) purified from rabbit skeletal muscle, chicken gizzard smooth muscle, bovine thyroid gland, and human platelet with similar $K_I$ values ($K_I = 2.5 \text{ \mu M}$). A detailed analysis of L-thyroxine inhibition of smooth muscle myosin light chain kinase activation was undertaken in order to determine the effect of L-thyroxine on the stoichiometries of Ca\textsuperscript{2+}, calmodulin, and the enzyme in the activation process. The kinetic data indicated that L-thyroxine does not interact with calmodulin but, instead, through direct association with the enzyme, inhibits the binding of the Ca\textsuperscript{2+}-calmodulin complex to MLC kinase. L-[\textsuperscript{125}I]-Thyroxine gel overlay revealed that the 95-kDa fragment of chicken gizzard MLC kinase digested by chymotrypsin and all the fragments of 110, 94, 70, and 43 kDa produced by \textit{Staphylococcus aureus} V8 protease digestion which contain the calmodulin binding domain retain L-[\textsuperscript{125}I]thyroxine binding activity, whereas smaller peptides were not radioactive. Since MLC kinase is phosphorylated by cAMP-dependent protein kinase (2 mol of phosphate/mol of MLC kinase), the effect of L-thyroxine on the phosphorylation of MLC kinase also was examined. L-Thyroxine binding did not inhibit the phosphorylation of MLC kinase and, moreover, reversed the inhibition of phosphorylation obtained with the calmodulin-enzyme complex. These observations support the suggestion that L-thyroxine binds at or near the calmodulin-binding site of MLC kinase. L-Thyroxine may serve as a different type of pharmacological tool for elucidating the biological significance of MLC kinase-mediated reactions.

Considerable attention has been directed to the mode of regulation of actin-myosin interactions, particularly in vertebrate smooth muscle and non-muscle cells. Most recent findings revealed that Ca\textsuperscript{2+}-calmodulin (CaM)-dependent phosphorylation of myosin light chains is a dominant regulatory mechanism. This reaction, which is obligatory for the stimulation of myosin ATP\textsubscript{ase}, is a prerequisite for tension development (Adelstein and Eisenberg, 1980; Walsh and Hartshorne, 1982).

Myosin light chain kinase (MLC kinase) was identified as an ATP\textsuperscript{-}-CaM-dependent enzyme which catalyzes the transfer of the $\gamma$-phosphate from ATP to the regulatory light chain of myosin (Dabrowska et al., 1978). Calmodulin antagonists such as the phenothiazine (trifluoperazine) and the N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) have been used as pharmacological tools to elucidate the function of Ca\textsuperscript{2+}. CaM-mediated systems both in vivo and in vitro (Weiss et al., 1974; Hidaka et al., 1978). However, calmodulin exerts a pleiotropic effect on many cellular functions. Therefore, in vivo studies employing "calmodulin antagonists" were inadequate to the elucidation of the role of the MLC kinase-mediated pathway. Recently, we synthesized novel MLC kinase inhibitors such as ML-9 and HA-140, which bind at or near the ATP binding site of MLC kinase (Saitoh et al., 1987; Hagiwara et al., 1987). Although these compounds exhibited a selective inhibition of MLC kinase not involving calmodulin, they also inhibit CaM-dependent protein kinase II. These results prompted us to search for a different type of MLC kinase inhibitor. We now report that L-thyroxine (T\textsubscript{4}) binds to the CaM-binding site of MLC kinase and can be used as a tool for elucidating the role of the enzyme both in vivo and in vitro.

**EXPERIMENTAL PROCEDURES**

[\textsuperscript{1}P]ATP and \textsuperscript{1}P]-[\textsuperscript{3}H]-Thyroxine were purchased from American. Chymotrypsin A\textsubscript{s} was from Boehringer Mannheim. \textit{Staphylococcus aureus} V8 protease and trypsin inhibitor (soybean) were from Sigma. DL-Thyroxine and DL-triiodothyronine were also purchased from Sigma. General laboratory reagents used were of analytical grade or better.

Calmodulin was purified from frozen bovine brain by the procedures described by Endo et al. (1981). Myosin light chain was prepared from chicken gizzard by the method of Hathaway and Haeberle (1983) and from rabbit skeletal muscle by the method of Blumenthal and Stull (1980). MLC kinase was purified from various tissues by the modified methods of Walsh et al. (1983) and Takio et al. (1985). The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart by the method of Beavo et al. (1974). Ca\textsuperscript{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) was prepared from rabbit brain, as described by Hidaka and Tanaka (1987). Casein kinase I from rat liver was prepared by the method of Meggio et al. (1979). Casein kinase II from rabbit skeletal muscle was prepared according to the method of Huang et al. (1982). Calmodulin kinase II was purified from rabbit brain by the procedure of Kennedy et al. (1983).

**Kinase Assays**—MLC kinase and other protein kinase activities were measured by quantification of [\textsuperscript{32}P]phosphate incorporation into isolated myosin light chain or other protein substrates, as described (Inagaki et al., 1986), in the absence or presence of thyroid hormones. Assays were performed for 1, 3, and 5 min at 30 °C and in all cases demonstrated a linear incorporation of [\textsuperscript{32}P]phosphate into the substrate over the 5-min assay. The reaction was terminated by the addition of 1 ml of ice-cold 20% trichloroacetic acid following addition of 600 µg of bovine serum albumin as a carrier protein. The sample was centrifuged at 3000 rpm for 15 min, the pellet was resuspended in ice-cold 10% trichloroacetic acid solution, and the centrifugation-
resuspension cycle was repeated three times. The final pellet was dissolved in 1 ml of 1 N NaOH, and radioactivity was measured by a liquid scintillation counter.

**Protein Digestion of Myosin Light Chain Kinase**—Limited proteolysis of chicken gizzard MLC kinase was carried out at 25 °C in 1.0 mM CaCl₂, 10 mM MgCl₂, and 20 mM Tris-HCl (pH 7.0). The final concentration of MLC kinase was 0.1 mg/ml, and reaction volume was 0.25 ml. The following protease concentrations were employed: α-chymotrypsin (1 μg/ml); S. aureus V8 protease (3 μg/ml). Aliquots (25 μl) of the reaction mixtures were withdrawn at t = 0, 5, 10, 15, 30, 60, and 120 min, added to an equal volume (25 μl) of NaDodSO₄ gel sample buffer (20 mM Tris-HCl (pH 6.8), 2% (w/v) NaDodSO₄, 30% (v/v) glycerol, 0.02% (w/v) bromphenol blue, and 2% (v/v) 2-mercaptoethanol) and immersed in a boiling water bath for 2 min. Samples (15 μl) were applied to gel lanes. The molecular weight marker proteins (Bio-Rad Laboratories) were electrophoresed on each slab gel. Electrophoresis was performed in 10% polyacrylamide slab gels with a 5% acrylamide stacking gel in the presence of 0.1% NaDodSO₄ at 30 mA, using the discontinuous buffer system of Laemmli (1970). Gels were stained in 45% (v/v) ethanol and 10% (v/v) acetic acid containing 0.14% (w/v) Coomassie Brilliant Blue R-250 and destained 10% (v/v) 2-propanol and 10% (v/v) acetic acid.

**Assessment of L-Thyroxine-binding Activity**—The L-thyroxine-binding activity of chick gizzard MLC kinase was determined by modifications of the 35S-calmodulin gel overlay described by Glenn and Weber (1980). NaDodSO₄-polyacrylamide gel procedure electrophoresis was performed using the discontinuous buffer system of Laemmli (1970), and the gel was fixed in 45% methanol, 10% acetic acid for 30 min. Gels were rinsed three times with distilled water and washed in 10% ethanol for 2 h at room temperature to remove the NaDodSO₄. They were then washed with 10% ethanol for 30 min and rinsed three times with water. The gels were subsequently incubated with 25 mM Tris-HCl (pH 7.0), 1 mM CaCl₂, and 10 mM MgCl₂ for 10 min and washed several times with the buffer. The gels were then incubated with gentle shaking for 1 h with the same solution containing L-[35S]thyroxine (10⁶ cpm/ml) at 25 °C. Unbound L-thyroxine was removed by repeated washings with the solution at 4 °C. The gels were washed briefly with distilled water, stained, dried, and subjected to autoradiography with Kodak X-Omat AR-x-ray film.

**RESULTS**

**Effect of Thyroid Hormone on Protein Kinases**—Effects of thyroid hormone, L-thyroxine, and 3,3',5'-triiodo-L-thyronine on phosphotransferase activities of casein kinase I, casein kinase II, cAMP-dependent protein kinase, protein kinase C, calmodulin kinase II, and MLC kinase were investigated. As shown in Fig. 1, L-thyroxine inhibited MLC kinase activity from chicken gizzard by 50% at the concentration of 9 × 10⁻⁶ M, while 3,3',5'-triiodo-L-thyronine produced 50% inhibition of the enzyme at the concentration of 2 × 10⁻⁵ M. In contrast to the potent ability of L-thyroxine to inhibit MLC kinases, much higher concentrations were necessary to inhibit the catalytic activities of casein kinase I, casein kinase II, cAMP-dependent protein kinase, protein kinase C, and calmodulin kinase II. In the case of thyroid hormone derivatives, DL-thyroxine and DL-triiodothyronine proved to be effective inhibitors, but 3,5-diodo-L-thyronine, L-thyroxine, 3,5-diodo-L-tyrosine, and DL-tyrosine did not affect MLC kinase activity up to 3 × 10⁻⁴ M (data not shown). There was no difference between the inhibitory properties of D and L isomers. As shown in Table 1, L-thyroxine inhibited the MLC kinase from chicken gizzard smooth muscle, rabbit skeletal muscle, bovine thyroid gland, and human platelet with similar Kᵢ values (about 2.5 μM).

**Kinetic Analysis of Inhibition Mechanism of MLC Kinase Activity**—Since the activation of MLC kinase requires the formation of the complex of Ca²⁺, calmodulin, and enzyme, we analyzed the effect of L-thyroxine on the stoichiometries of the activation process. Fig. 24 illustrates the activation of MLC kinase by calmodulin at various fixed concentrations of L-thyroxine. As the L-thyroxine concentration was increased, the enzyme was activated by higher concentrations of calmodulin.

![FIG. 1. Effect of L-thyroxine on various protein kinases.](image)

The maximal rate of 3²P incorporation by each protein kinase was determined in the absence of L-thyroxine. Details of reaction conditions for individual experiments are as follows. MLC kinase activity (C) was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 0.1 mM calcium chloride, 15 mM CaM, 5-100 μM [γ-³²P]ATP (4 × 10⁶ cpm), 10 μM smooth or skeletal muscle myosin light chain, and 0.1 μg/ml MLC kinase (specific activity, 5 μmol/min/mg). Protein kinase C activity (D) was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 0.5 mM calcium chloride, 25 μM phosphatidylserine, 10 μM [γ-³²P]ATP (4 × 10⁶ cpm), 100 μg of histone H₁, and 1.5 μg/ml of the enzyme (specific activity, 0.12 μmol/min/mg). Calmodulin kinase II activity (E) was assayed in a reaction mixture containing 100 mM calmodulin, 10 μM [γ-³²P]ATP, 5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM EGTA, 0.7 mM CaCl₂, 10 mM Hepes (pH 7.5), 0.25 M NaOH, and radioactivity was measured by a liquid scintillation counter.

**Table I**

| Tissue source of MLC kinase | Kᵢ (μM) |
|-----------------------------|---------|
| Chicken gizzard             | 2.5     |
| Rabbit skeletal muscle      | 2.4     |
| Bovine thyroid gland        | 2.4     |
| Human platelet              | 2.5     |
Inhibition of MLC Kinase by $T_4$

**FIG. 2.** A, Effect of L-thyroxine concentration on MLC kinase activation by calmodulin. The rates of $^{32}$P incorporation into 20-kDa chicken gizzard myosin light chains were determined at the indicated concentrations of calmodulin and L-thyroxine. The 20-kDa light chain, MLC kinase, and Ca$^2+$ concentrations are 6 $\mu$M, 0.2 $\mu$g/ml, and 100 $\mu$M, respectively. B, Hill plots of calmodulin activation curves. Values for the Hill coefficient and $K_{CM_{app}}$ as a function of L-thyroxine were determined from the slope and horizontal axis intercept ($\log [v/(V_{max} - V)] = 0$), respectively. The same $V_{max}$ value was used in the calculation of position with respect to the vertical axis for all points. Best-fit lines were determined by least squares linear regression analysis.

**FIG. 3.** A, Effect of L-thyroxine concentration on the activation of MLC kinase by Ca$^2+$. Assays were performed as described under "Experimental Procedures" in the presence of 3 nM calmodulin, 6 $\mu$M 20-kDa light chain, 0.2 $\mu$g/ml MLC kinase, and the indicated concentrations of L-thyroxine. Ca$^2+$ concentrations less than 10 $\mu$M Ca$^2+$ were controlled by using a Ca$^2+/EGTA$ buffer system as described by Blumenthal and Stull (1980). Ca$^2+$ concentrations greater than 10 $\mu$M were not buffered by EGTA. B, Hill plots of Ca$^2+$ activation curves. Data from panel A were analyzed to determine values for the Hill coefficient and $K_{Ca^{2+}}$ from the slope and value of $\log [v/(V_{max} - V)] = 0$, respectively, for each Ca$^2+$ activation curve. The values of $V_{max}$ used to calculate the position of a given point with respect to the vertical axis were 100, 67, and 24% of the maximal rate for the curves determined at 0 (O), 10 (●), and 30 (△) $\mu$M L-thyroxine, respectively. Best-fit lines were determined by least squares linear regression analysis.

In contrast to the calmodulin activation curves, the apparent $V_{max}$ of a given Ca$^2+$ activation curve apparently decreased as the L-thyroxine concentration was increased (Fig. 3A). Analysis of the Ca$^2+$ activation curves by means of Hill plots (Fig. 3B) resulted in a Hill coefficient of 3 at any L-thyroxine concentration. These data suggest that L-thyroxine competitively inhibits the calmodulin binding to the enzyme.

**L-[${}^{35}$S]Thyroxine Gel Overlay**—To determine the L-thyroxine-binding site of MLC kinase, L-[${}^{35}$S]thyroxine binding to digested fragments of MLC kinase was examined. Chicken gizzard MLC kinase (unphosphorylated) was digested in the absence of CaM with chymotrypsin or S. aureus V8 protease. The time courses of protease digestion patterns by gel electrophoresis (Figs. 4A and 5A) and autoradiography (Figs. 4B and 5B) were presented. The time course of the digestion of MLC kinase by 1 $\mu$g/ml chymotrypsin is shown in Fig. 4A. The major product was a 95-kDa fragment, although prolonged digestion produced smaller fragments of 60 and 24

modulin; i.e. the calmodulin concentration required for half-maximal activation ($K_{CM_{app}}$) increased. The same $V_{max}$ values were obtained for all the calmodulin activation curves. When the calmodulin activation curves were analyzed by Hill plots (Fig. 2B), the Hill coefficient of 1 was not affected by L-thyroxine.

In contrast to the calmodulin activation curves, the apparent $V_{max}$ of a given Ca$^2+$ activation curve apparently decreased as the L-thyroxine concentration was increased (Fig. 3A). Analysis of the Ca$^2+$ activation curves by means of Hill plots (Fig. 3B) resulted in a Hill coefficient of 3 at any L-thyroxine concentration. These data suggest that L-thyroxine competitively inhibits the calmodulin binding to the enzyme.
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**Fig. 4.** L-[^125]I]Thyroxine gel overlay of α-chymotrypsin-digested MLC kinase. Digests were performed at 25 °C as described under "Experimental Procedures"; final concentrations in the mixture were 0.1 mg/ml MLC kinase, 1 mM CaCl$_2$, 10 mM MgCl$_2$, 25 mM Tris-HCl (pH 7.0) and α-chymotrypsin, 1 µg/ml. The digest mixtures were subjected to electrophoresis on 10% NaDodSO$_4$-polyacrylamide gel (1.0µg of MLC kinase/lane), and L-thyroxine binding was assessed by means of the gel overlay procedure described under "Experimental Procedures." Panel A, Coomassie staining. Panel B, L-[^125]I]thyroxine gel overlay.

**Fig. 5.** L-[^125]I]Thyroxine gel overlay of S. aureus V8 protease-digested MLC kinase. Three µg/ml S. aureus V8 protease was used for MLC kinase digestion. Other conditions are the same as Fig. 5. Panel A, Coomassie staining; panel B, L-[^125]I]thyroxine gel overlay.

kDa. The autoradiograms in Fig. 4B showed that all the 125I radioactivity was recovered in the 95-kDa fragment. As can be seen in Fig. 5A, V8 protease resulted in a sequential cleavage pattern with major fragments appearing at 110, 94, and 70 kDa with most of the protein accumulating eventually in the 70-kDa fragment. At the 2-h time point, there was also a band at 50 kDa. All these fragments retained L-[^125]I]thyroxine-binding activity (Fig. 5B).

**Phosphorylation Sites of MLC Kinase**—The time course of phosphorylation of chicken gizzard MLC kinase by the catalytic subunit of cAMP-dependent protein kinase is shown in Fig. 6. In the absence of CaM, 1.8 mol of phosphate/mol of MLC kinase were incorporated, while in the presence of Ca$^{2+}$. CaM, only 1.0 mol of phosphate/mol of MLC kinase was incorporated by cAMP-dependent protein kinase (Fig. 6A). These results demonstrate that one site of phosphorylation is blocked by CaM binding, as originally reported by Conti and Adelstein (1981). However, after a 10-min incubation of MLC kinase with 50 µM L-thyroxine at 25 °C, 1.7 mol of phosphate/mol of MLC kinase were incorporated even in the presence of CaM (Fig. 6B). The accessibility of the two phosphorylation sites evidently reflects complete dissociation of the MLC kinase-calcium complex in the presence of excess L-thyroxine.

**DISCUSSION**

In smooth muscle (Dabrowska et al., 1977) and non-muscle cells (Nishikawa et al., 1980; Trotter and Adelstein, 1979), MLC kinase is important for cellular events related to change in shape and secretory processes. This protein kinase requires both Ca$^{2+}$ and calmodulin for full activity (Dabrowska et al., 1978). Tawata et al. (1983) noted the existence of an MLC kinase in the thyroid gland that shares many of the characteristics of gizzard MLC kinase. Kobayashi et al. (1979) had previously reported the presence of calmodulin in the human thyroid. Our finding that thyroxine and triiodothyronine bind to MLC kinase and inhibit the calmodulin activation of the enzyme may relate to the elevated levels of thyroid calmodulin identified in patients with Graves' disease (Segal et al., 1984).

In previous works using a CaM antagonist, W-7, and the MLC kinase inhibitors, ML-9 and HA-140, we showed that MLC kinase plays a major role in the regulation of contractile proteins by phosphorylating the 20,000-dalton light chain of myosin in vascular smooth muscle (Hidaka et al., 1978; Saitoh et al., 1987), and blood platelets (Nishikawa et al., 1980; Hagiwara et al., 1987). Calmodulin antagonists including naphthalenesulfonamides (Hidaka et al., 1978), phenothiazines (Norman et al., 1979), and imidazoliums (Gietzen et al., 1981) inhibit Ca$^{2+}$-calmodulin-dependent protein phosphorylation systems through interactions with Ca$^{2+}$-calmodulin. The thyroxine-induced inhibition of MLC kinase also could be overcome by increasing the concentrations of calmodulin. Thyroxine had no effect on Ca$^{2+}$-calmodulin-independent MLC kinase digested by trypsin (Hagiwara et al., 1988). However, interaction between thyroid hormone and calmodulin was ruled out in measurements of the dialyzability of the labeled thyroid hormone in the presence of purified calmodulin and various concentrations of Ca$^{2+}$ (Davis and Davis, 1995). We reported that derivatives of W-7, such as HA-140 (Hagiwara et al., 1987) and ML-9 (Saitoh et al., 1987), inhibited MLC kinase in a competitive fashion with respect to ATP and suggested that these compounds bind at or near the ATP-binding site of the enzyme. However, the inhibition of
MLC kinase by thyroxine was not competitive with ATP (data not shown). Evidently, that inhibitory mechanism of L-thyroxine is different from HA-140 or ML-9.

In this report, we performed a kinetic analysis of the L-thyroxine inhibition mechanism. The Hill coefficients of chicken gizzard MLC kinase of 1 for calmodulin activation and of 3 for Ca\(^{2+}\) activation are comparable with those of skeletal MLC kinase determined by Blumenthal and Stull (1980). The calmodulin activation curves are shifted to the right by increasing L-thyroxine concentration with no change in the Hill coefficient, suggesting that the L-thyroxine-binding site overlaps the calmodulin-binding site of the enzyme. Increased accessibility of the phosphorylation site of MLC kinase, which is normally blocked in the presence of calmodulin, reinforces the conclusion that the enzyme-calmodulin complex dissociates in solutions containing excess L-thyroxine. The 70-kDa and 43-kDa fragments generated by S. aureus V8 protease digestion and the 95-kDa fragments generated by chymotrypsin cleavage retain both CaM-binding (Foyt et al., 1985; Walsh, 1985) and L-[\(^{35}S\)]thyroxine-binding activity as determined by the gel overlay techniques. The thyroxine-binding site is apparently not located in the active site, since thyroxine-induced inhibition is not competitive with either ATP or the myosin light chain. The binding of thyroxine to the tryptic fragment of MLC kinase does not affect its catalytic activity (Hagiwara et al., 1988). Additionally, the thyroxine-binding site is probably distinct from the phosphorylation sites recognized by Ca\(^{2+}\)-dependent protein kinase since thyroxine does not affect the phosphorylation obtained in the absence of calmodulin. In all, these results indicate that L-thyroxine is a different type of MLC kinase inhibitor which binds directly to the calmodulin binding site of the enzyme. In this regard, we have successfully conducted the affinity purification of MLC kinase from human platelets using L-thyroxine as an affinity ligand. From the viewpoint of molecular pharmacology, L-thyroxine may be a useful probe for studies of the physiological functions of MLC kinase.

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