Vol. 152, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1988 Pages 270-276

Thyroid Hormones Inhibit the Ca^{2+} Calmodulin-Induced Activation of Myosin Light Chain Kinase

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Received March 7, 1988

L-Thyroxine (T_4) and L-triiodothyronine (T_3) specifically, inhibited myosin light chain kinase (MLC-kinase) from various tissues whereas inhibitory effects of T_4 and T_3 on other protein kinases such as protein kinase C, cAMP-dependent protein kinase, casein kinase I, casein kinase II and calmodulin kinase II were much weaker. T_4 was a more potent inhibitor of MLC-kinase than T_3. Kinetic studies showed that T_4 behaved as a competitive inhibitor of MLC-kinase toward calmodulin (CaM) and that Ki value was 2.5 μM. The activity of the catalytic fragment of MLC-kinase, which is active without CaM, was not inhibited by T_4. ^{125}I-T_4 gel overlay revealed that CaM did not bind T_4 but MLC-kinase had ^{125}I-T_4 binding activity. These observations suggest that T_4 binds at or near CaM binding domain of MLC-kinase and inhibits CaM-induced activation of MLC-kinase.

In smooth muscle (1) and non-muscle cells (2), MLC-kinase is important for cellular events related to change in shape and secretory processes. This protein kinase requires both Ca^{2+} and CaM for full activity. In previous works using a CaM-antagonist; W-7 and the MLC-kinase inhibitor; ML-9, 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 (3, 4) and blood platelets (2). In the thyroid gland, Tawata et al. (5) noted the existence of MLC-kinase that

The abbreviations used are: MLC-kinase, myosin light chain kinase; CaM, calmodulin; EGTA, ethyleneglycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid.

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0006-291X/88 $1.50

shares many of the characteristics observed in gizzard MLC-kinase. Kobayashi et al. (6) reported the presence of CaM in the human thyroid, and CaM levels may be increased in Graves' disease (7). Our data revealed that thyroxine and triiodothyronine bound to MLC-kinase and inhibited the CaM-activation of the enzyme.

EXPERIMENTAL PROCEDURES

[γ-^{32}P]ATP and L-[3',5'-^{125}I]thyroxine were purchased from Amersham Corp. TPCK-trypsin (bovine pancreas), and trypsin inhibitor (soybean) were from Sigma Chemical Co. D,L-thyroxine, D,L-triiodothyronine, and D,L-tyrosine were also purchased from Sigma. Other thyroid hormone derivatives were generous gifts of Prof. A. Nagasaka (Fujita Gakuen Univ.). 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. (8). Myosin light chain was prepared from chicken gizzard by the method of Hathaway and Haeberle (9) and from rabbit skeletal muscle by the method of Blumenthal and Stull (10). MLC-kinase was purified from various tissues by the modified methods of Walsh et al. (11) and Takio et al. (12). The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart by the method of Beavo et al. (13). Ca^{2+}-activated, phospholipid dependent protein kinase (protein kinase C) was prepared from rabbit brain, as described by Hidaka and Tanaka (14). Casein kinase I from rat liver was prepared by the method of Meggio et al. (15). Casein kinase II from rabbit skeletal muscle was prepared according to the method of Huang et al. (16). Calmodulin kinase II was purified from rabbit brain by the procedure of Kennedy et al. (17). MLC-kinase and other protein kinase activities were measured by quantification of [^{32}P]phosphate incorporation into isolated myosin light chain or other protein substrates, as described (18), in the absence or presence of thyroid hormones.

The L-thyroxine-binding activity of chicken gizzard MLC-kinase was determined by modifications of the ^{125}I-calmodulin gel overlay described by Glenny and Weber (19). SDS-polyacrylamide gel procedure electrophoresis was performed using the discontinuous buffer system of Laemmli (20) and the gel fixed in 40% methanol, 7% acetic acid for 30 min. Gels were rinsed three times with distilled water and washed in 10% ethanol for 2 hr at room temperature to remove the SDS. 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 $CaCl_2$ and 10 mM $MgCl_2$ for 10 min and washed several times with the buffer. The gels were then incubated with gentle shaking for 1 hr with the same solution containing ^{125}I-L-thyroxine (10^6 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 AND DISCUSSION

Effects of thyroid hormone, L-thyroxine and 3,5,3'-triiodo-L-thyronine on phosphotransferase activities of casein kinase I, casein kinase II, cAMP-dependent protein kinase, protein kinase C, calmodulin kinase II and

Table I. Inhibition of various protein kinase activities by L-thyroxine (T_4) and 3,5,3'-triiodo-L-thyronine (T_3)

MLC-kinase	Concentration of I_{50} (μM)	
	T_4	T_3
Casein Kinase I	420	>1000
Casein Kinase II	310	210
cAMP-dependent protein kinase	130	>1000
Protein Kinase C	110	120
CaM kinase II	190	86
MLC-kinase (Chicken gizzard)	8.9 ± 2.0	22.1 ± 4.0
MLC-kinase (Skeletal muscle)	8.7 ± 1.8	19.8 ± 3.0
MLC-kinase (Thyroid gland)	6.7 ± 1.1	15.1 ± 3.1

I_{50}; inhibitor concentration which cause 50% inhibition of enzyme activity. IC_{50} values producing inhibition of MLC-kinase from various tissues are means of two independent determinations. The assay procedures of protein kinases are described under "Experimental Procedures".

MLC-kinase were investigated. As shown in Table 1, L-thyroxine inhibited MLC-kinase activities from chicken gizzard, bovine thyroid gland and rabbit skeletal muscle by 50% at the concentration of 9×10^{-6} M, while 3,5,3'-triiodo-L-thyronine produced 50% inhibition of the enzymes at the concentration of 2×10^{-5} M. Contrary to potent abilities of thyroid hormone to inhibit MLC-kinases, enzyme activities of casein kinase I, casein kinase II, cAMP-dependent protein kinase, protein kinase C, and calmodulin kinase II were affected only weakly by L-thyroxine and 3,5,3'-triiodo-L-thyronine. The inhibition of chicken gizzard MLC-kinase by thyroid hormones and their derivatives were examined (Table II).

Table II

Inhibition of MLC-kinase activity by thyroid hormone and its derivatives

Inhibitors	Concentration of I_{50} (μM)
L-Thyroxine	*8.9 ± 2.0
D-Thyroxine	*8.3 ± 1.6
L-Triiodothyronine	*22.1 ± 4.0
D-Triiodothyronine	19.5 ± 3.3
L-Diiodothyronine	>100
L-thyronine	>100
L-Diiodotyrosine	>100
L-tyrosine	>100
D-tyrosine	>100

I_{50}; inhibitor concentration which causes 50% inhibition of chicken gizzard MLC-kinase activity. The assay was performed in the presence of saturating amounts of Ca^{2+} (0.1 mM) and CaM (0.4 μg/ml). The values presented are means of two independent determinations. * are included in Table I.

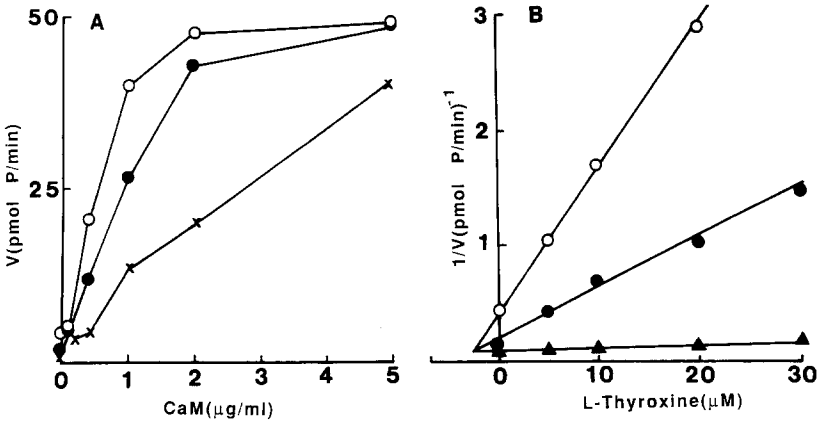

Fig. 1.
A. Effect of increasing concentrations of CaM on thyroxine-induced inhibition of chicken gizzard MLC-kinase. L-Thyroxine concentrations were 0 μM (○), 10 μM (●), and 30 μM (×), respectively.
B. Kinetic analysis of thyroxine-induced inhibition of chicken gizzard MLC-kinase when the data were plotted as 1/V vs. L-thyroxine concentration. The assay was performed in the presence of 0.1 mM Ca^{2+} and 0.05 μg/ml (○), 0.2 μg/ml (●), and 2 μg/ml (▲) of CaM.

D,L-thyroxine and D,L-triiodothyronine proved to be effective inhibitors, but 3,5-diiodo-L-thyronine, L-thyronine, 3,5-diiodo-L-tyrosine and D,L-tyrosine did not affect MLC-kinase activity up to 3×10^{-4} M. There was no difference between the ability of the D and L form to inhibit the MLC-kinase activity.

Since the activation of MLC-kinase requires calmodulin (CaM), we determined whether or not increasing concentrations of CaM would prevent the L-thyroxine-induced inhibition of MLC-kinase. This L-thyroxine-induced inhibition of the enzyme could be overcome by high concentrations of CaM (Fig. 1A), but not by increasing the concentrations of myosin light chains or ATP used (data not shown). Kinetic studies showed that L-thyroxine behaved as a competitive inhibitor of MLC-kinase toward CaM and the Ki value was calculated to be 2.5 μM (Fig. 1B).

MLC-kinase was reported to be alternatively activated in an irreversible manner by limited proteolysis with trypsin (21). In this process, the catalytically active fragment produced was entirely independent of Ca^{2+} and CaM. The Ca^{2+}-CaM-independent activity of MLC-kinase was not inhibited by L-thyroxine up to 3×10^{-5} M (Fig. 2), while Ca^{2+}-CaM-dependent MLC-kinase

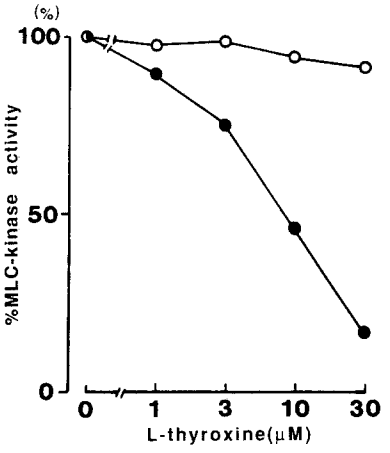

Fig. 2. Effect of L-thyroxine on Ca^{2+}-CaM activated (●) and trypsin digested (○) MLC-kinase activity. Ca^{2+}-CaM activated chicken gizzard MLC-kinase activity was determined in the presence of 0.1 mM $CaCl_2$ and 0.4 μg/ml CaM, and the assay of digested MLC-kinase was performed in the presence of 2 mM EGTA. The MLC-kinase activity determined in the absence of L-thyroxine was shown as a control (100%) activity.

activity was depressed in a dose-dependent fashion. In the light of all these findings, the inhibitory action of L-thyroxine seems to be due to the enzyme-activating process of MLC-kinase by Ca^{2+}-CaM.

To determine the target protein of L-thyroxine in MLC-kinase inhibition, the L-thyroxine-binding activity of CaM and MLC-kinase was assessed by means of gel overlay technique. MLC-kinase (Mr 130,000)(Fig. 3,

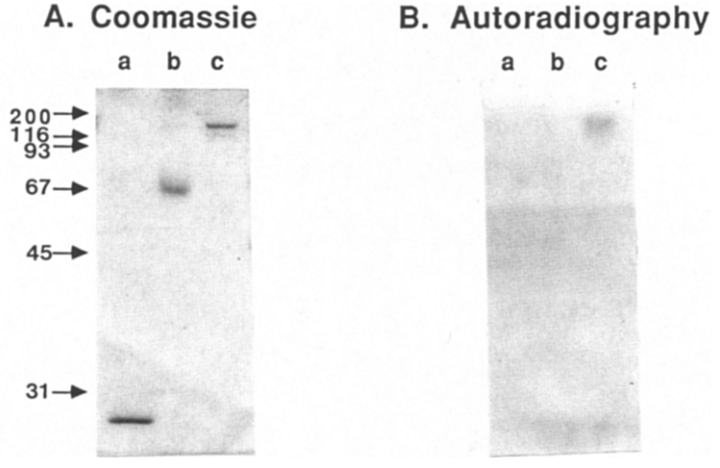

Fig. 3. ^{125}I-L-thyroxine gel overlay of CaM and MLC-kinase. Panel A, Coomassie staining. Panel B, Autoradiography. 2 μg of CaM (lane a), bovin serum albumin (lane b) and chicken gizzard MLC-kinase (lane c), were subjected to electrophoresis on a 15% SDS-PAGE. ^{125}I-thyroxine gel overlay was performed as described under "Experimental Procedures".

lane C) showed ^{125}I-L-thyroxine-binding activity, whereas CaM (Mr 16,000) or albumin bind little or no thyroxine. These results suggest that thyroxine affects the activation process of MLC-kinase by Ca^{2+}-CaM. Interaction between thyroid hormone and CaM was excluded by dialysis of the labeled thyroid hormone in the presence of purified CaM and various concentrations of Ca^{2+} (22). Our ^{125}I-thyroxine gel overlay (Fig. 3) supported this finding and indicated that thyroxine binds directly to MLC-kinase. We reported that derivatives of W-7, such as A-3 (18) and ML-9 (3) inhibited MLC-kinase in a competitive fashion with ATP, and suggested that these compounds bind at or near the ATP-binding site of the enzyme. The inhibition of MLC-kinase by thyroxine was not competitive with ATP. All these results indicate that thyroxine is a unique type of MLC-kinase inhibitor. The inhibitory action of thyroxine seems to be the result of direct effect on the CaM-binding site of the enzyme.

ACKNOWLEDGMENTS

We thank Dr. A. Nagasaka (Fujita Gakuen Univ.) for providing thyroid hormone and its derivatives and M. Ohara (Kyushu Univ.) for comments on the manuscript.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and by a research grant for cardiovascular disease from the Ministry of Health and Welfare.

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