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J Clin Invest. 1983;71(3):579-586. https://doi.org/10.1172/JCI110803.

Research Article

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Role of Calmodulin in Thyroid Hormone Stimulation In Vitro of Human Erythrocyte Ca^{2+}-ATPase Activity

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ABSTRACT Because human erythrocyte membrane Ca^{2+}-ATPase is a calmodulin-dependent enzyme, and because physiological levels of thyroid hormone stimulate this enzyme system in vitro, we have studied the role of calmodulin in this model of extranuclear thyroid hormone action. Ca^{2+}-ATPase activity in the absence of thyroid hormone ("basal activity") was increased by inclusion in the preassay incubation mixture of purified calmodulin or hypothyroid erythrocyte hemolysate that contained calmodulin (39 μg calmodulin/ml packed cells, determined by radioimmunoassay); addition of L-thyroxine or 3,5,3′-triiodo-L-thyronine (10^{-10} M) significantly enhanced ($P < 0.001$) enzyme activity in the presence of calmodulin or hemolysate. The stimulatory effects of thyroid hormone, calmodulin, and hemolysate were additive. At 5–10 μM, trifluoperazine, an antagonist of calmodulin, inhibited thyroid hormone stimulation of Ca^{2+}-ATPase activity. Higher concentrations of trifluoperazine (50–100 μM) inhibited basal and hormone-stimulated enzyme activity, with or without added calmodulin. Anti-calmodulin antibody (10–50 μg antibody/mg membrane protein) inhibited basal, calmodulin-stimulated and thyroid hormone-stimulated Ca^{2+}-ATPase activity. Membrane preparations were shown by radioimmunoassay to contain residual endogenous calmodulin (0.27±0.02 μg/mg membrane protein). The latter accounts for the effect of trifluoperazine and calmodulin antibody on membrane Ca^{2+}-ATPase activity in the absence of added purified calmodulin. These results support the conclusion that the in vitro action of physiological levels of iodothyronines on human erythrocyte Ca^{2+}-ATPase activity requires the presence of calmodulin.

INTRODUCTION

The stimulation in vitro by physiological concentrations of thyroid hormone (10^{-10} to 10^{-12} M) of human erythrocyte Ca^{2+}-stimulated, Mg^{2+}-dependent adenosine triphosphatase (Ca^{2+}-ATPase)[1] activity has recently been described by this laboratory (1). The action of L-thyroxine (T_4) on this membrane transport-associated enzyme does not require prior conversion of T_4 to 3,5,3′-triiodo-L-thyronine (T_3) (1). The effect of iodothyronines in this system is species-dependent (2) and is specific for T_4, T_3 and certain structural analogs, such as 3,5-dimethyl-3′-isopropyl-L-thyronine (DIMIT) (3). Iodothyronines such as 3,3′,5′-L-T_3 (reverse T_3), 3,5,3′-triiodothyroacetic acid, D-T_3 and D-T_4 (3) do not stimulate Ca^{2+}-ATPase activity. Because Ca^{2+}-ATPase in the human erythrocyte is a calmodulin-dependent enzyme (4), we have conducted a series of in vitro studies to determine the role of calmodulin in this extranuclear action of thyroid hormone.

METHODS

Erythrocyte membranes. Heparinized blood samples were obtained from euthyroid human volunteers by a protocol approved by the institutional Human Research Committee. Washed erythrocyte membranes ("ghosts") were prepared in hypotonic Tris buffer (10 mM) in the presence of EDTA (0.1 mM), as previously described (1). This method results in membranes with low residual calmodulin content (4). Ghosts were stored for 24–72 h at −70°C until used. Previous studies have shown that the washing process removes >99% of endogenous thyroid hormone bound to erythrocyte membranes (1).

In a separate series of experiments, erythrocyte membranes were prepared in imidazole buffer by the method of Jarrett and Penniston (5).

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Received for publication 11 November 1981 and in revised form 23 November 1982.

[1] *Abbreviations used in this paper*: Ca^{2+}-ATPase, Ca^{2+}-stimulated, Mg^{2+}-dependent, adenosine triphosphatase; TFP, trifluoperazine; T_4, L-thyroxine; T_3, 3,5,3′-triiodo-L-thyronine.

Hormones and reagents. T_4, T_3, and Na_2ATP were purchased from Sigma Chemical Co. (St. Louis, MO). Trifluoperazine was kindly supplied by Smith, Kline and French Co. (Philadelphia, PA). Purified rat testis calmodulin, sheep anti-calmodulin antibody (lot numbers 122 011 281, 188 011 281) and all reagents for calmodulin radioimmunoassay were obtained from CAABCO (Houston, TX).

Preparation of erythrocyte hemolysate. Erythrocytes obtained from patients with untreated primary hypothyroidism were pelleted, resuspended in an equal volume of 10 mM Tris buffer, pH 7.4, and lysed by sonication (Branson Sonicator, Danbury, CT; iced samples subjected to 20-s passes ×3 at 40 W). Radioimmunoassay of T_4 (6) and T_3 (7) in hemolysate revealed no detectable iodothyronine. Protein content of the hemolysate was 150 mg/ml, determined by the Lowry method (8), with bovine serum albumin (BSA) as standard. Hemolysate was stored at −70°C for up to 6 mo before use.

Incubation of membranes prior to Ca^{2+}-ATPase assay. Suspensions of erythrocyte membranes (1 mg membrane protein/ml buffer [1]) were incubated at 37°C for 60 min prior to enzyme assay. In various experiments, hemolysate (2–10 μl/ml), calmodulin (20–200 ng/ml), trifluoperazine (TFP) (5–100 μM), or anticalmodulin antibody (10–50 μg/ml) were included in the incubation mixture, as were, where indicated, T_4 or T_3 in concentrations ranging from 10^{-4} to 10^{-14} M.

Ca^{2+}-ATPase assay. Ca^{2+}-ATPase activity was measured by the method of Strittmatter et al. (9), as modified in our laboratory (1), in which enzyme activity is calculated as the difference in inorganic phosphate liberated from Na_2ATP in the presence and absence of 0.15 mM $CaCl_2$. Inorganic phosphate (P_i) was measured by the Fiske-Subbarow method (10) and enzyme activity expressed as micromoles P_i/milligram membrane protein/90-minute assay period. All studies were carried out in duplicate in two or more experiments. Results are expressed as means±SE of Ca^{2+}-ATPase activity. Intraassay coefficient of variation of measurements of Ca^{2+}-ATPase activity was ±0.7%. There was as much as 30% interassay variation due to changes in basal membrane activity with freezing and thawing of membrane preparations from individual donors. Erythrocytes from each donor provided sufficient membranes for one or two complete experiments; each experiment was conducted with membranes from a single donor.

In each assay were included blanks for nonenzymatic hydrolysis of ATP and liberation of phosphate from erythrocyte membranes. In experiments involving hemolysate, background phosphate content of these fractions was subtracted from ATP hydrolysis measurements.

In selected studies, the Ca^{2+}-ATPase activity of erythrocyte membranes was assayed according to the method of Jarrett and Penniston (5). In this assay, carried out in imidazole buffer, enzyme activity is measured as the difference between micromoles P_i liberated/milligram membrane protein/15-min assay period, in the presence and absence of calcium.

Calmodulin radioimmunoassay. The calmodulin content of solubilized erythrocyte membranes and hypothyroid hemolysate was determined by radioimmunoassay according to the method of Chafouleas et al. (11), utilizing 0.5 ml Triton X-100/500 ml of homogenization and assay buffers to avoid gel formation in the hemolysate.

Statistical significance of the thyroid hormone effect was determined by paired *t* test. The enzyme activity of each sample incubated with T_4 was compared with that of a simultaneous control sample without T_4 (basal activity), and the significance of the difference between the two activity levels (that is, the change with T_4, or ΔT_4) was determined.

RESULTS

Stimulation of human erythrocyte Ca^{2+}-ATPase activity in vitro by iodothyronines. Basal erythrocyte membrane Ca^{2+}-ATPase activity was 0.206±0.027 μmol P_i/mg per 90 min (Table I). This level of activity approximates that reported by Strittmatter et al. (9), from whose method our technique derives. These activity data, obtained in the absence of thyroid hormone, are based on studies conducted on membranes from nine euthyroid donors.

The addition of T_4 (10^{-10} M) to membranes resulted in a mean Ca^{2+}-ATPase activity of 0.273 μmol P_i/mg per 90 min, which is an increase in enzyme activity (ΔT_4) of 0.067± 0.006 over basal levels measured in paired samples ($P < 0.001$) (Table I). The absolute increase in Ca^{2+}-ATPase activity obtained with thyroid hormone was in part determined by the level of basal membrane enzyme activity ($r = 0.72$, $P < 0.01$, abso-

TABLE I

Effect of Hypothyroid Hemolysate and Purified Calmodulin on Erythrocyte Ca^{2+}-ATPase Activity

Hypothyroid hemolysate‡	Calmodulin§	Ca^{2+}-ATPase activity, μmol $P_i \cdot mg^{-1} \cdot 90\ min^{-1}$*			
		Basal	T_4, 10^{-10} M	ΔT_4	P‖
−	−	0.206±0.027	0.273±0.032	+0.067±0.006	<0.001
+	−	0.372±0.048	0.460±0.056	+0.088±0.011	<0.001
−	+	0.279±0.038	0.347±0.041	+0.068±0.006	<0.001

* Results are means±SE of 14 experiments in which determinations were made in duplicate. The assay was performed as described in Methods, using 1 mg membrane protein/ml. Basal activity represents activity in the absence of thyroid hormone; ΔT_4 indicates change in enzyme activity with addition of T_4, 10^{-10} M.

‡ Hemolysate concentration was 10 μl/mg membrane protein.

§ Calmodulin concentration was 100 ng/mg membrane protein.

‖ *P* was calculated by paired *t* test to estimate significance of thyroid hormone effect (ΔT_4).

lute increase in Ca^{2+}-ATPase activity with T_4 vs. basal level).

In experiments carried out with T_3 (10^{-10} M), similar stimulatory effects on Ca^{2+}-ATPase activity were obtained (data not shown). We have previously reported that T_3 is slightly less active on a molar basis than T_4 in this human cell enzyme system (1, 3, 12).

Because basal enzyme activity can be materially affected by the method of membrane preparation (4), we compared the Ca^{2+}-ATPase activity of membranes prepared by our method (Tris buffer) with the activity of erythrocyte membranes prepared by the method of Jarrett and Penniston (5); both sets of membranes were obtained from the same donor and assayed in the same experiment. The technique of Jarrett and Penniston depends upon hypotonic lysis of cells in water, followed by membrane washes in imidazole buffer. Comparison of the enzyme activities obtained from the two preparations is shown in Table II, part A, and indicates that the imidazole-prepared membranes have approximately 2.5-fold the basal activity of Tris-prepared ghosts when both sets of membranes are assayed for Ca^{2+}-ATPase in Tris. Significant stimulation of membrane enzyme activity by T_4 is observed when membranes are prepared in imidazole and assayed in Tris. However, use of imidazole buffer in the enzyme assay system, when ghosts were prepared in Tris, resulted in no basal Ca^{2+}-ATPase activity and no thyroid hormone-stimulability of the enzyme (Table II, part B). It should be noted that preparation of membranes in imidazole in our hands, coupled with enzyme activity assay in imidazole, did result in basal activity comparable to that reported by Jarrett and Penniston (5), namely, 0.555 μmol P_i/mg per 15 min, or 37.0 nmol P_i/mg per min.

Effect of erythrocyte hemolysate on Ca^{2+}-ATPase activity. In the absence of added thyroid hormone, incubation of hypothyroid erythrocyte hemolysate with ghosts (10 μl hemolysate/mg membrane protein) resulted in significant enhancement of Ca^{2+}-ATPase activity over hemolysate-free control (increase in activity, 0.166±0.029 μmol P_i/mg per 90 min, $P < 0.001$) (Table I). The hemolysate contained no endogenous T_4 or T_3 detectable by sensitive radioimmunoassay. When T_4 (10^{-10} M) was added to erythrocyte ghosts in the presence of hemolysate, an increase in enzyme activity of 0.088±0.011 was obtained, compared to enzyme activity without added T_4.

Table III shows results of a dose-response study of T_4 in the presence and absence of hemolysate. The change in enzyme activity with progressive additions of T_4 described a parabolic dose-response relationship of thyroid hormone and Ca^{2+}-ATPase activity over a range of thyroid hormone concentrations of 10^{-4} to 10^{-14} M. Maximal hormonal effect was obtained in the absence and presence of hemolysate at 10^{-10} M T_4. The action of T_3 on Ca^{2+}-ATPase was also maximal at a T_3 concentration of 10^{-10} M in both the absence and presence of hemolysate (data not shown). It should be pointed out that interpretation of dose-response studies

TABLE II

Comparison of Effects of Methods of Membrane Preparation and Enzyme Assay on Determination of Human Erythrocyte Ca^{2+}-ATPase Activity

					Ca^{2+}-ATPase activity*		
	Membranes‡		Assay§		Basal	T_4, 10^{-10} M	
	Imidazole	Tris	Imidazole	Tris	μmol $P_i \cdot mg^{-1} \cdot 90\ min^{-1}$		P‖
A	−	+	−	+	0.176±0.012	0.238±0.013	<0.001
	+	−	−	+	0.455±0.048	0.495±0.044	<0.001
					μmol $P_i \cdot mg^{-1} \cdot 15\ min^{-1}$		
B	−	+	+	−	0	0	
	+	−	+	−	0.555±0.074	0.425±0.112	NS

* Results are means±SE of three experiments in which determinations were carried out in duplicate. Enzyme activity is expressed as micromoles P_i liberated/milligram membrane protein/90 min or /15 min (5), as indicated. Basal Ca^{2+}-ATPase activity refers to activity measured in the absence of added T_4.

‡ Membranes were prepared in imidazole buffer by the method of Jarrett and Penniston (5) or in Tris buffer by our previously described technique (1).

§ Ca^{2+}-ATPase assay was performed in imidazole buffer by the method of Jarrett and Penniston (5) or in Tris buffer by our previously reported method (1).

‖ P was calculated by paired t test to estimate significance of the thyroid hormone effect.

TABLE III

Effect of Hypothyroid Hemolysate on Thyroid Hormone Stimulation of Erythrocyte Ca^{2+}-ATPase Activity

T_4	Ca^{2+}-ATPase activity, μmol $P_i \cdot mg^{-1} \cdot 90\ min^{-1}$*			
	Without hemolysate	ΔT_4‡	Hemolysate§	ΔT_4
M				
—	0.150±0.015	—	0.264±0.033	—
10^{-4}	0.160±0.015	+0.010 (NS)	0.299±0.024	+0.035 (<0.05)
10^{-8}	0.217±0.036	+0.067 (<0.05)	0.351±0.036	+0.087 (<0.001)
10^{-10}	0.261±0.020	+0.111 (<0.01)	0.387±0.027	+0.123 (<0.001)
10^{-14}	0.160±0.015	+0.010 (NS)	0.217±0.013	−0.047 (NS)

* Results are means±SE of two experiments in which determinations were made in duplicate. The assay was performed as described in Methods, using 1 mg membrane protein/ml.

‡ ΔT_4 represents the change in Ca^{2+}-ATPase activity with T_4, compared to basal activity without T_4. Figures in parentheses are *P* values calculated by paired *t* test, comparing enzyme activity in the absence of T_4 with activity in the presence of T_4 in concentrations from 10^{-14} to 10^{-4} M. NS indicates $P > 0.05$.

§ Hemolysate concentration was 10 μl/mg membrane protein.

involving low concentrations of thyroid hormone ($<10^{-10}$ M) is made difficult by the presence of hemoglobin-containing hemolysate; we have reported elsewhere that hemoglobin binds iodothyronines (13).

Effect of added calmodulin on Ca^{2+}-ATPase activity. Addition to membranes of purified calmodulin in the absence of exogenous thyroid hormone increased Ca^{2+}-ATPase activity ($P < 0.001$, Table I). Maximal calmodulin response was obtained at a concentration of 100 ng/mg membrane protein (Fig. 1). In the presence of added calmodulin, a significant stimulatory effect of T_4 on the enzyme was also observed (Table I; Fig. 1) and was additive to the action of calmodulin. The use of animal source calmodulin was justified because no interspecies differences in calmodulin structure have been discerned (11).

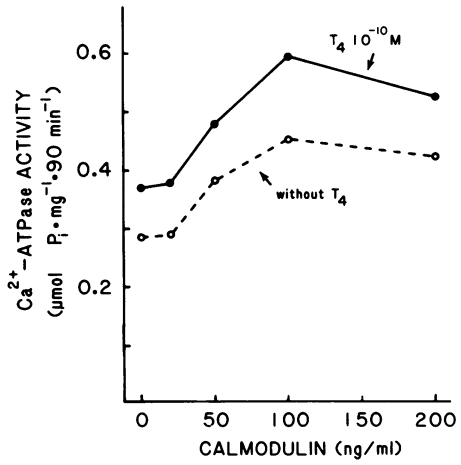

FIGURE 1 The effect of exogenous calmodulin on Ca^{2+}-ATPase activity in human erythrocyte membranes, with and without added T_4. The enzyme assay was carried out as described in Methods, with calmodulin concentrations of 0–200 ng/mg membrane protein per ml assay volume. The data represent the mean values of enzyme activity in duplicate samples from three experiments.

When purified calmodulin (100 ng/mg membrane protein) and hypothyroid hemolysate (10 μl/mg membrane protein) were concurrently incubated with erythrocyte membranes, the stimulatory effects of these two agents were additive in both the absence and presence of T_4 (Table IV). That is, Ca^{2+}-ATPase activity obtained with optimal concentrations of calmodulin and T_4 was further enhanced when hemolysate was present. Thus, hypothyroid hemolysate may contain enzyme stimulatory factors in addition to calmodulin.

Effect of TFP on T_4 stimulation in vitro of Ca^{2+}-ATPase activity. Concentrations of TFP of 50–100 μM were required to definitively reduce basal Ca^{2+}-ATPase activity of membranes (Fig. 2). However, low concentrations of TFP (5 and 10 μM) inhibited the T_4 effect without decreasing basal enzyme activity. This selective inhibitory action was observed in the presence of purified calmodulin (Fig. 2B) and when hemolysate was added to membranes (Table V). In the presence of hemolysate, as little as 5 μM TFP reduced hormonal stimulation of enzyme activity to a barely detectable level (0.016 μmol P_i/mg per 90 min), whereas 100 μM TFP was needed to materially decrease basal enzyme activity (Table V).

Effect of calmodulin antibody on stimulation in vitro of Ca^{2+}-ATPase activity by calmodulin, T_4, and hypothyroid hemolysate. In the absence of exogenous

TABLE IV

Additive Effect of Hypothyroid Hemolysate and Purified Calmodulin on Erythrocyte Ca^{2+}-ATPase Activity

Hypothyroid hemolysate‡	Calmodulin§	Ca^{2+}-ATPase activity, μmol $P_i \cdot mg^{-1} \cdot 90\ min^{-1}$* Basal	T_4, 10^{-10} M	P‖
−	−	0.331±0.035	0.444±0.028	<0.001
−	+	0.413±0.049	0.499±0.039	<0.01
+	−	0.530±0.039	0.602±0.039	<0.001
+	+	0.609±0.077	0.657±0.060	<0.05

* Results are means±SE of three experiments in which determinations were made in duplicate. The assay was performed as described in Methods, using 1 mg membrane protein/ml. Basal activity represents enzyme activity in the absence of thyroid hormone.
‡ Hemolysate concentration was 10 μl/mg membrane protein.
§ Calmodulin concentration was 100 ng/mg membrane protein.
‖ *P* was calculated by paired *t* test to estimate significance of difference in enzyme activity with and without T_4.

calmodulin, antibody to calmodulin in a concentration of 50 μg/mg membrane protein reduced basal Ca^{2+}-ATPase activity from 0.365 μmol P_i/mg per 90 min to 0.202 μmol (Table VI). The antibody also prevented the stimulatory effect of thyroid hormone on erythrocyte membrane Ca^{2+}-ATPase (Table VI). These studies were repeated in the presence of exogenous calmodulin (100 ng/mg membrane protein), using an antibody:calmodulin ratio of 50:1 (antibody, 150,000 D, calmodulin, 16,700 D [14]). Basal Ca^{2+}-ATPase activity was lowered in the presence of antibody, but a

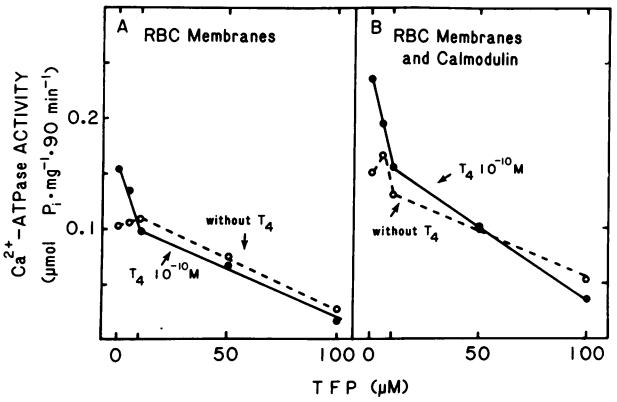

FIGURE 2 The effect of TFP on Ca^{2+}-ATPase activity in erythrocyte membranes, with and without added T_4 and calmodulin. The enzyme assay was carried out as described in Methods, in the presence or absence of T_4, 10^{-10} M. Panels A and B show enzyme activity in the absence and presence, respectively, of calmodulin, 100 ng/mg membrane protein per ml assay volume. TFP concentrations were 0, 5, 10, 50, and 100 μM. The data represent the mean values of enzyme activity in duplicate samples from three experiments.

TABLE V

Effect of TFP on Stimulation of Erythrocyte Ca^{2+}-ATPase Activity by Hypothyroid Hemolysate and Thyroid Hormone

TFP	Ca^{2+}-ATPase activity, μmol $P_i \cdot mg^{-1} \cdot 90\ min^{-1}$* Basal	ΔT_4	P‡
μM			
	0.227±0.018	+0.055±0.003	<0.001
5	0.257±0.001	+0.016±0.014	NS
10	0.233±0.003	+0.038±0.022	NS
50	0.206±0.000	+0.013±0.005	NS
100	0.052±0.000	−0.003±0.006	NS

* Results are means±SE of results from two experiments in which determinations were made in duplicate. The enzyme assay was performed as described in Methods, using 1 mg membrane protein/ml and 10 μl hypothyroid hemolysate/mg membrane protein. Basal activity refers to activity with hemolysate but in the absence of added thyroid hormone. ΔT_4 represents the change in enzyme activity obtained with the addition of T_4, 10^{-10} M.
‡ *P* was calculated by paired *t* test to estimate the significance of ΔT_4.

TABLE VI

Effect of Calmodulin Antibody on Thyroid Hormone Stimulation of Ca^{2+}-ATPase Activity

Calmodulin‡	Calmodulin antibody§	Ca^{2+}-ATPase activity, μmol $P_i \cdot mg^{-1} \cdot 90\ min^{-1}$* Basal	ΔT_4	P‖
−	−	0.365±0.031	+0.110±0.008	<0.001
−	+	0.202±0.029	+0.017±0.011	NS
+	−	0.436±0.037	+0.087±0.013	<0.001
+	+	0.242±0.038	+0.004±0.005	NS

* Results are means±SE of four experiments in which determinations were made in duplicate. The enzyme assay was performed as described in Methods, using 1 mg membrane protein/ml. Shown are enzyme activities in the absence of T_4 ("basal") and the change in enzyme activity in the presence of T_4, 10^{-10} M (ΔT_4).
‡ Calmodulin concentration was 100 ng/mg membrane protein.
§ Calmodulin antibody concentration was 50 μg/mg membrane protein.
‖ *P* was calculated by paired *t* test to estimate significance of thyroid hormone effect. In additional experiments the albumin content of the calmodulin antibody preparation was shown not to significantly inhibit either basal or T_4-stimulated Ca^{2+}-ATPase activity. Without T_4, basal activity in the absence of calmodulin was 0.341 and 0.316 μmol P_i/mg per 90 min (*P* = NS by paired *t* test) with and without albumin, respectively. In the presence of calmodulin, basal Ca^{2+}-ATPase activity was 0.424 and 0.408 (*P* = NS), without and with albumin, respectively. With T_4, 10^{-10} M, added to the system, Ca^{2+}-ATPase activity was 0.431 and 0.416 (*P* = NS), without and with albumin.

stimulatory action of exogenous calmodulin persisted (Table VI). The additive stimulation of enzyme ordinarily provided by T_4 in the presence of purified calmodulin was not expressed when antibody was present at this molar ratio (Table VI).

To further define the antibody effect in this membrane system, we titrated the inhibitory effect of antibody on stimulation of Ca^{2+}-ATPase by calmodulin and T_4 (Fig. 3). In the absence of antibody, the expected stimulation by T_4 of Ca^{2+}-ATPase was observed (Fig. 3, vertical panel). With the addition of antibody at 10 μg/mg membrane protein (= 10 μg/ml incubation mixture), basal Ca^{2+}-ATPase was reduced and there was no enhancement of enzyme activity by T_4. Coincubation of membranes with antibody and purified calmodulin (100 ng/mg membrane protein; molar ratio of antibody:calmodulin, 10:1) resulted in partial restoration of basal membrane activity, as well as T_4-stimulated activity, to levels achieved in the absence of antibody (Fig. 3). When the antibody:calmodulin ratio was reduced to 5:1 (calmodulin concentration, 200 ng/mg membrane protein), there was full restoration of basal Ca^{2+}-ATPase activity and further expression of the additive thyroid hormone effect on the enzyme. When Table VI and Fig. 3 are compared, differences in the effective ratio of antibody:calmodulin

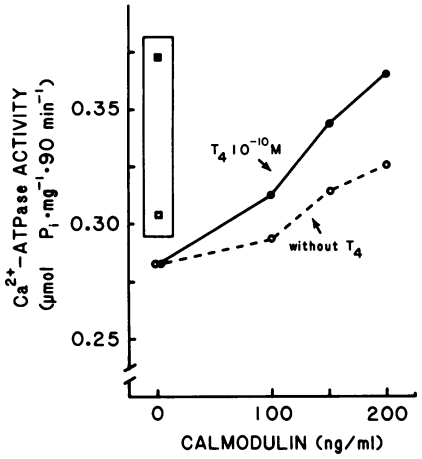

FIGURE 3 Inhibition by calmodulin antibody of basal erythrocyte membrane Ca^{2+}-ATPase activity and of iodothyronine stimulation of Ca^{2+}-ATPase: effect of addition of purified calmodulin. The enzyme assay was performed as described in Methods. Calmodulin concentrations ranged from 0 to 200 ng/mg membrane protein per ml assay volume and antibody concentration was 10 μg/mg membrane protein. Basal (□) and T_4-stimulated (■) Ca^{2+}-ATPase activity, in the absence of antibody or purified calmodulin, is shown in the vertical panel. Enzyme activity in the presence of antibody is shown graphically in the absence (O - - - O) and presence (● —— ●) of T_4, 10^{-10} M. Data are means of enzyme activity from duplicate determinations in three experiments.

are observed; this is attributable to the use of erythrocyte membranes of varying content of residual endogenous calmodulin (see below) and to the use of two batches of calmodulin antibody. These studies show that the antibody inhibits the action of calmodulin on erythrocyte membrane Ca^{2+}-ATPase and concomitantly prevents T_4 stimulation of the enzyme. Thus, hormonal stimulation of Ca^{2+}-ATPase is a calmodulin-dependent function.

The effect of calmodulin antibody on stimulation of Ca^{2+}-ATPase activity by hemolysate was also examined. In the absence of antibody, enzyme activity showed the expected increase when exposure of membranes to hypothyroid hemolysate occurred (2 μl hemolysate/mg membrane protein), rising to 0.320 μmol P_i/mg per 90 min with hemolysate from 0.272 μmol P_i. In the presence of antibody (50 μg/mg membrane protein), the stimulatory effect of hemolysate was also observed, albeit at lower membrane activity levels (0.232 μmol P_i with hemolysate, 0.186 μmol P_i without hemolysate). At this particular ratio of antibody:hemolysate, results were similar to those reported in antibody-calmodulin studies (Table VI).

The antibody preparation contained bovine serum albumin (1 μg/μg antibody). In separate studies we found that albumin, alone, in a concentration comparable to that in the antibody studies described above, did not alter Ca^{2+}-ATPase activity in the absence or presence of either calmodulin or T_4 (see footnote, Table VI).

Calmodulin antibody was also studied to determine if it contained protein capable of binding iodothyronine. Charcoal precipitation of unbound labeled T_4 in a solution of antibody (10 μg/ml) and [^{125}I]T_4 showed that no protein binding of T_4 occurred. Dialysis of a solution of antibody and tracer showed no retention of hormone by protein in the dialysand.

Calmodulin content of erythrocyte membranes and hypothyroid hemolysate. The residual endogenous calmodulin content of 16 samples of human erythrocyte membranes, prepared hypotonically according to our previously reported method (1), was 0.27±0.02 (SE) μg/mg membrane protein, measured by radioimmunoassay. The range was 0.14 to 0.44 μg/mg membrane protein. Calmodulin concentration in the hypothyroid hemolysate was 19.5 μg/ml (39.0 μg/ml packed cells), so that the calmodulin content of 10 μl of hemolysate was 0.195 μg.

DISCUSSION

Ca^{2+}-ATPase in the human erythrocyte is a calcium pump-associated enzyme that maintains intracellular calcium concentration at a low level (15) and is responsive in vitro to physiological concentrations of thy-

roid hormone. Human erythrocyte Ca^{2+}-ATPase activity is a model of extranuclear hormone action with inherent advantages over animal cell models that require nanomolar or higher concentrations of iodothyronines to demonstrate actions on transport (16–18) or maintenance of donor animals on high saturated fat intake prior to ATPase measurements (19). The present studies examined the hypothesis that calmodulin, the activator protein for Ca^{2+}-ATPase (4, 5, 20) and a regulatory protein for several intracellular enzymes (21), is essential to the expression of thyroid hormone action on Ca^{2+}-ATPase activity.

TFP is a phenothiazine known to inhibit the action of calmodulin (22), presumably by binding to the cytoplasmic calmodulin · Ca^{2+} complex which activates membrane-bound Ca^{2+}-ATPase. The addition of TFP to erythrocyte membranes in the present studies eliminated the iodothyronine effect on Ca^{2+}-ATPase and reduced basal enzyme activity in both the presence and absence of purified calmodulin. These observations are consistent with a postulated requirement for calmodulin in hormonal stimulation of Ca^{2+}-ATPase activity and also suggested that our erythrocyte membranes contained residual endogenous calmodulin activity. Recent evidence, however, has shown that TFP is not a wholly specific inhibitor of actions of calmodulin (23). Results we have obtained[2] in fact indicate that TFP, in concentrations of 5–10 μM, blocks thyroid hormone-binding to membranes, whereas at 50–100 μM levels the agent appears to more generally inhibit calmodulin-dependent events, such as Ca^{2+}-ATPase activity, as shown in Fig. 2.

More specific evidence in support of the dependence on calmodulin of thyroid hormone action in the human erythrocyte model was provided by studies carried out with calmodulin antibody. In the absence of thyroid hormone, calmodulin antibody partially inhibited Ca^{2+}-ATPase activity in erythrocyte ghosts (Table VI), consistent with the presence of endogenous calmodulin in membranes. Calmodulin radioimmunoassay of the membranes subsequently confirmed that small quantities of calmodulin persist in our hypotonically prepared ghosts. The addition of purified calmodulin to antibody-membrane mixtures overcame the suppressive action of antibody on enzyme activity (Table VI). Stimulation of Ca^{2+}-ATPase activity by T_4 was inhibited by antibody, and this inhibition was reversed by the addition of progressively larger quantities of purified calmodulin in the presence of a fixed amount of antibody (Fig. 3). These observations strongly support the thesis that the iodothyronine effect can be expressed only in the presence of active calmodulin. Additional studies showed that the antibody effect on thyroid hormone action could not be attributed to the binding of hormone by antibody protein.

Elsewhere we have shown that thyroid hormone and calmodulin do not directly interact (12), so that it is not likely that stimulation of Ca^{2+}-ATPase activity in vitro by thyroid hormone involves an alteration in the nature of the calmodulin · Ca^{2+} complex. On the basis of the observations reported here and studies of the binding of iodothyronines by erythrocyte membranes (12), we believe that stimulation by thyroid hormone of erythrocyte Ca^{2+}-ATPase activity requires (*a*) the presence of membrane-associated calmodulin · Ca^{2+} complex, as described by Scharff (20), and (*b*) the interaction of thyroid hormone with the cell membrane.

Our erythrocyte model is based on the preparation of membranes that are calmodulin poor (4) and are thought to approximate endogenous basal state conditions. The method of membrane preparation and assay for Ca^{2+}-ATPase activity in Tris buffer used in the current studies results in basal enzyme activity estimates that are similar in magnitude to those reported by Strittmatter et al. (9) from which our techniques were derived. Our membrane Ca^{2+}-ATPase activity levels, however, are low compared to results from other methods (4, 5, 15). The factors that contribute to the differences in enzyme activity include buffer composition both at the time of membrane preparation and at enzyme assay. Farrance and Vincenzi (24) have previously shown that Tris buffer leads to the expression of lower Ca^{2+}-ATPase activities than are observed in the presence of imidazole or histidine buffers (24) used in other laboratories (5, 25). The stimulatory effect of T_4 on Ca^{2+}-ATPase is demonstrable when the enzyme assay is carried out in Tris (Table II), regardless of whether erythrocyte membranes are prepared in Tris or imidazole. However, the in vitro hormone effect is entirely lost when imidazole is used in the enzyme assay. We have recently found that thyroid hormone stimulates calcium efflux from intact human erythrocytes (26), supporting the functional significance of iodothyronine stimulation of Ca^{2+}-ATPase activity in vitro; these efflux experiments were carried out with cells maintained in Tris buffer.

When purified calmodulin and hypothyroid hemolysate were added together to erythrocyte membranes, additive stimulation of Ca^{2+}-ATPase activity was observed. This was the case despite the fact that calmodulin was used at a concentration (100 ng/ml; 6×10^{-9} M) shown to be maximally effective in our laboratory and by others (5). This suggests that hemolysate contains a stimulator of enzyme activity that is distinct from calmodulin; a noncalmodulin activator has been described in membrane preparations by Mauldin and Roufogalis (27).

[2] Davis, F. B., P. J. Davis, and S. D. Blas. Manuscript in preparation.

ACKNOWLEDGMENTS

The expert technical assistance of Mrs. Marion Schoenl is gratefully acknowledged. Maria Zielezny, Ph.D. provided statistical support, and Mrs. Barbara Lenahan provided secretarial assistance in the preparation of the manuscript.

This work was supported in part by National Institutes of Health research grant AM 26113.

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