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Ca^{2+}-STIMULATED, Mg^{2+}-DEPENDENT ATPase IN BOVINE THYROID PLASMA MEMBRANES

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An isolated plasma membrane fraction from bovine thyroid glands contained a Ca^{2+}-stimulated, Mg^{2+}-dependent adenosine triphosphatase ((Ca^{2+} + Mg^{2+})-ATPase) activity which was purified in parallel to (Na^+ + K^+)-ATPase and adenylate cyclase. The (Ca^{2+} + Mg^{2+})-ATPase activity was maximally stimulated by approx. 200 μM added calcium in the presence of approx. 200 μM EGTA (69.7 ± 5.2 nmol/mg protein per min). In EGTA-washed membranes, the enzyme was stimulated by calmodulin and inhibited by trifluoperazine.

Calcium ions participate in a variety of cellular activities [1–6]. Control of intracellular free Ca^{2+} concentration thus appears to be crucial for the maintenance of normal cell function. There are two known mechanisms by which the plasma membrane of a cell maintains the low (about 0.1 μM) intracellular free Ca^{2+} concentration as compared to the much higher extracellular concentration (1 mM). One employs an ATP-utilizing calcium pump, which involves hydrolysis of ATP by a (Ca^{2+} + Mg^{2+})-ATPase. This calcium pump has been well characterized in the erythrocyte plasma membrane [7–9]. Similar (Ca^{2+} + Mg^{2+})-ATPases have also been identified in plasma membranes from brain [10], adipocytes [11], pancreatic islets [12], macrophages [13], neutrophils [14], corpus luteum [15] and cardiac sarcolemma [16], and have been implicated in the calcium pump in these tissues. Another mechanism is an Na^+ gradient-driven Ca^{2+} extrusion [17,18] as described in the case of excitable tissues.

The effects of thyrotropin on the thyroid are mediated mainly by the generation of cyclic AMP [19,20]. However, regulation of Ca^{2+} may have important metabolic effects. The thyrotropin stimulation of [1-^{14}C]glucose oxidation, $^{32}P_i$ incorporation into phospholipids [21] and iodide organification (unpublished data) is dependent upon the presence of Ca^{2+} in the buffer. Furthermore, acetylcholine and ionophore A23187 induce several metabolic changes via an increase of Ca^{2+} flux in the cell, some of which mimic and others which antagonize those of thyrotropin [22,23]. The present paper describes the presence and some properties of a (Ca^{2+} + Mg^{2+})-ATPase in bovine thyroid plasma membranes.

Plasma membranes were prepared from bovine thyroid glands [24,25]. The precipitate from the final centrifugation was suspended in a small volume of 0.24 M sucrose (pH 7.4) and used immediately for enzyme assays (except adenylate cyclase) or stored in aliquots in plastic tubes at −20°C. Enzyme activities were determined in triplicate by the respective method described in Table I [26–31]. Since the activity of (Ca^{2+} + Mg^{2+})-ATPase decreased progressively during storage at −20°C, this enzyme assay was performed within

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

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TABLE I

SPECIFIC ACTIVITY OF MARKER ENZYMES AND OF ATPases IN HOMOGENATE AND PLASMA MEMBRANE FRACTION OF BOVINE THYROID

ATPase was assayed in 0.5 ml buffer containing 25 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 2 mM ATP, 200 μM EGTA (buffer A) and enzyme fraction at 37 C for 15 to 20 min by monitoring the ^{32}P released from [γ-^{32}P]ATP [26] except that the phosphomolybdate complex was extracted into an isobutanol-benzene (1 : 1) mixture. Two ml of the 3 ml organic phase was mixed with 3 ml of scintillator and counted. Basal Mg^{2+}-ATPase was measured in the buffer A. ($Na^+ + K^+$)-ATPase was measured by subtracting the values obtained in the buffer A containing 10 mM NaN_3 from those in the buffer A containing 100 mM NaCl, 10 mM KCl and 10 mM NaN_3. ($Ca^{2+} + Mg^{2+}$)-ATPase was measured by subtracting values obtained in the buffer A containing 10 mM NaN_3 from those in the buffer A containing 200 μM $CaCl_2$ and 10 mM NaN_3. K^+-ATPase measured in the presence of EDTA activity (K^+-ATPase (EDTA)) was determined (27) in the buffer containing 25 mM Tris-maleate, pH 7.0, 2 mM ATP, 2 mM EDTA and 0.6 M KCl. 5'-Nucleotidase was assayed at 37 C for 15 min [28]. The P_i in the acid-supernatant by terminating with trichloroacetic acid (final concentration 5%) was determined [29]. Adenylate cyclase activity was measured [30] with slight modification [31]. In these assays, 100–200 μg of homogenate protein and 10–30 μg of plasma membrane protein were used. The values represent mean ± S.E. TSH, thyrotropic hormone.

Enzyme activity	N	Homogenate	Plasma membranes	Purification fold
Mg^{2+}-ATPase	4	10.1 ± 2.0 [a]	239.7 ± 39.5	23.7
($Ca^{2+} + Mg^{2+}$)-ATPase	4	0.9 ± 0.1 [a]	69.7 ± 5.2	77.4
($Na^+ + K^+$)-ATPase	4	4.8 ± 0.7 [a]	360.8 ± 41.5	75.2
K^+-ATPase (EDTA)	3	4.1 ± 3.1 [a]	27.4 ± 5.3	6.7
5'-Nucleotidase	4	4.0 ± 0.4 [a]	65.3 ± 3.4	16.3
Adenylate cyclase				
Basal		10.5 ± 3.9 [b]	175.1 ± 49.9	16.7
TSH 10 mU/ml		27.6 ± 7.4 [b]	1084.7 ± 143.2	39.3
NaF 10 mM		24.1 ± 4.8 [b]	1021.9 ± 93.2	42.4

[a] Enzyme activity expressed in nmol/mg protein per min.
[b] Enzyme activity expressed in pmol/mg protein per 5 min.

one week after preparation of the plasma membranes. This enzyme activity was linear for at least 30 min when ATP hydrolysis was less than 15% and with 10–50 μg of plasma membrane protein. Calmodulin was prepared from bovine testes [32]. Protein was measured with bovine serum albumin as standard [33]. [γ-^{32}P]ATP (10–40 Ci/mmol) and [α-^{32}P]ATP (410 Ci/mmol) were obtained from Amersham-Searle, and cyclic [^3H]AMP (32.3 Ci/mmol) was from New England Nuclear. All solutions were made up in distilled water passed through an ion exchange column. Bovine thyrotropic hormone (B-9, 21.1 U/mg) and trifluoperazine were kindly provided by the NIAMDD and by Smith, Kline and French, respectively.

As shown in Table I, the fraction collected from the 30/41% sucrose interface was enriched in enzymes characteristic of plasma membranes. The thyrotropic hormone and NaF responsive adenylate cyclase activities were increased approx. 40-fold while 5'-nucleotidase activity was increased 16-fold. The Mg^{2+}-ATPase and ($Na^+ + K^+$)-ATPase, respectively, exhibited a 23.7- and 75.2-fold enrichment in the plasma membrane fraction. The ($Ca^{2+} + Mg^{2+}$)-ATPase activity assayed with 200 μM added Ca^{2+} and 200 μM EGTA was enriched 77.4-fold. In contrast, the activity of K^+-ATPase measured in the presence of EDTA, characteristic of myosin [27], increased only 6.7-fold. The ($Ca^{2+} + Mg^{2+}$)-ATPase activity was not modified by 20 mM NaCl, KCl or 0.5 mM ouabain or the exclusion of 10 mM NaN_3 (Table III). Since mitochondrial ATPase is inhibited by 10 mM NaN_3 [34] and the high affinity ($Ca^{2+} + Mg^{2+}$)-ATPase from the endoplasmic reticulum of adipocytes required both Mg^{2+} and K^+ [35], these results suggest that it is different from the mitochondrial or the endoplasmic reticulum enzyme. The possibility that the ($Ca^{2+} + Mg^{2+}$)-ATPase reaction is catalyzed by membrane-bound myosin [36] is unlikely, because the specific activity of K^+-ATPase measured in the presence of EDTA increased in

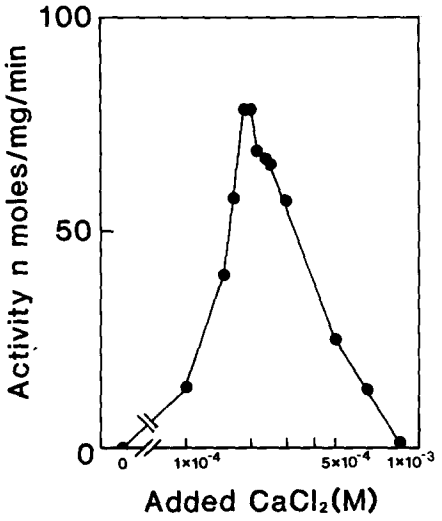

Fig. 1. Effect of added calcium on $(Ca^{2+}+Mg^{2+})$-ATPase of bovine thyroid plasma membranes. The enzyme activity was assayed at 100–1000 μM added calcium in the presence of 200 μM EGTA.

TABLE II

EFFECT OF VARIOUS AGENTS AND CALMODULIN ON $(Ca^{2+}+Mg^{2+})$-ATPase ACTIVITY

$(Ca^{2+}+Mg^{2+})$-ATPase activity was assayed as described in Table I (Complete condition) at 200 μM added calcium and 200 μM EGTA in the presence or absence of various agents and calmodulin. The data shown are mean ± S.E. of enzyme activity assayed in triplicate.

Addition or subtraction	Specific activity (% of control)
Complete	100.0 ± 4.7
+20 mM NaCl	116.2 ± 6.1
+ 0.5 mM ouabain	103.3 ± 2.5
−10 mM NaN_3	108.4 ± 7.0
−10 mM NaN_3 + 20 mM KCl	
0.5 mM ouabain	99.3 ± 2.6
+ 4 μg calmodulin	94.8 ± 2.4

the plasma membranes to a much lesser extent than those of $(Na^{+}+K^{+})$-ATPase, adenylate cyclase and $(Ca^{2+}+Mg^{2+})$-ATPase.

In the presence of 200 μM EGTA, activation of the $(Ca^{2+}+Mg^{2+})$-ATPase gradually increased to a maximum at approx. 200 μM (Fig. 1). Above this concentration the activity decreased with no stimulation at 1000 μM. With added Ca^{2+} at $\leqslant 170$ μM, the free Ca^{2+} level in the reaction mixture was estimated to be in the order of $\leqslant \mu$M, according to the $(Ca)_{total}/(EGTA)_{total}$ ratio [37]. Furthermore, the enzyme activity was stimulated by the initial addition of calcium (200 μM) in the presence of 200 μM EGTA to 650.5 nmol/mg protein at 15 min at which time an excess (final concentration of 400 μM) of EGTA was added. The enzyme activity remained constant until the readdition of calcium at 30 min (providing a final concentration of 450 μM) when the enzyme activity was again immediately stimulated reaching 1108.7 nmol/mg at 45 min from 631.0 nmol/mg at 30 min. The results demonstrate that the stimulation of the $(Ca^{2+}+Mg^{2+})$-ATPase activity by calcium and its inhibition by EGTA are readily reversible [38].

In the untreated plasma membranes, 4 μg of calmodulin had no effect on the enzyme activity (Table II). In contrast, as shown in Fig. 2 the

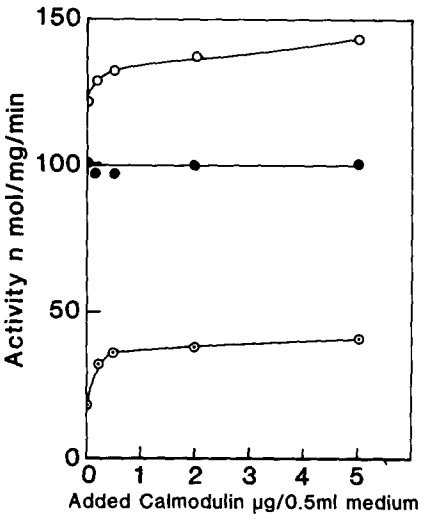

Fig. 2. Dependence of $(Ca^{2+}+Mg^{2+})$-ATPase on added calmodulin. Plasma membranes stored in 0.24 M sucrose (6–10 mg/ml) were thawed and then incubated at 4°C for 15 min in 25 mM Tris-HCl, pH 7.4, containing 5 mM EGTA. The incubation mixture was diluted with ice-cold buffer (final concentration 1 mM EGTA) and centrifuged at 30000×g for 20 min at 4°C. The pellet was dissolved in the same buffer and again centrifuged. The $(Ca^{2+}+Mg^{2+})$-ATPase was assayed in the presence of 200 μM EGTA and 200 μM added calcium with the addition of calmodulin after appropriate dilution with cold water. ●——●, Basal Mg^{2+}-ATPase; ⊙——⊙, $(Ca^{2+}+Mg^{2+})$-ATPase; ○——○, total ATPase.

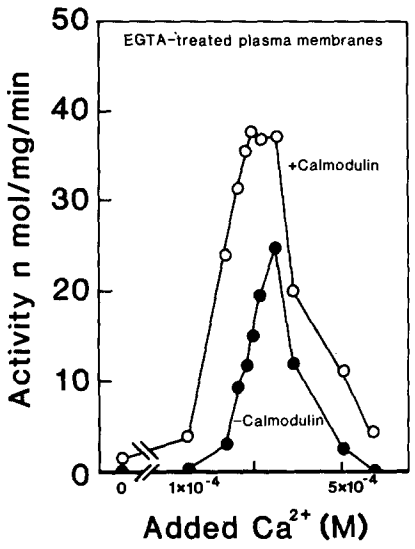

Fig. 3. Effects of added calcium and calmodulin on the $(Ca^{2+} + Mg^{2+})$-ATPase of plasma membranes which had been treated with 5 mM EGTA as described in Fig. 2. The enzyme activity was measured in the presence of 200 μM EGTA and added calcium as indicated with (○) or without (●) the addition of 4 μg calmodulin.

addition of 5 μg of calmodulin maximally stimulated (200%) the enzyme activity in the 5 mM EGTA-treated plasma membranes with no significant effect on the basal Mg^{2+}-ATPase. Such treatment has been reported to remove bound calmodulin in other tissues [10,16]. Fig. 3 also shows the dependence of the $(Ca^{2+} + Mg^{2+})$-ATPase activity in the EGTA-treated membranes on the addition of both calcium and calmodulin. In the presence of 200 μM EGTA, 150–500 μM calcium activated the enzyme. Four μg of calmodulin increased the enzyme activity over the entire range where calcium was stimulatory. Calmodulin increased both the maximum rate and the affinity for Ca^{2+} of the enzyme. Phenothiazine drugs inhibit the calmodulin stimulation of phosphodiesterase and $(Ca^{2+} + Mg^{2+})$-ATPase [39,40]. Trifluoperazine inhibited the $(Ca^{2+} + Mg^{2+})$-ATPase activity in both membrane preparations with or without EGTA treatment. Mg^{2+}-ATPase was also slightly inhibited. The K_i value of the drug on the $(Ca^{2+} + Mg^{2+})$-ATPase activities in both preparations was 30–60 μM. These results suggest that the $(Ca^{2+} + Mg^{2+})$-ATPase in the thyroid plasma membrane may be regulated by endogenous calmodulin [41,42], since calmodulin has been identified in the thyroid [43]. However, the present results differ from those of Verma and Penniston [15] who found that the high affinity $(Ca^{2+} + Mg^{2+})$-ATPase of corpus luteum plasma membranes was not stimulated by calmodulin or inhibited by trifluoperazine. They reported that this failure might be related to the high content of tightly bound calmodulin in the membranes, because much of the calmodulin bound to the membranes could not be extracted even by washing with 1 mM EGTA and/or 0.1% (w/v) Triton X-100.

Since the $(Ca^{2+} + Mg^{2+})$-ATPase in the thyroid plasma membrane is similar to the high affinity $(Ca^{2+} + Mg^{2+})$-ATPase in plasma membranes of erythrocytes and some other tissues [7–10,16], it is quite possible that the enzyme in the thyroid is involved in the maintenance of the appropriate intracellular Ca^{2+} concentration for the regulation of calcium-dependent metabolic changes in the cell.

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