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Synthesis of ATP catalyzed by the (Ca^{2+} + Mg^{2+})-ATPase from erythrocyte ghosts. Energy conservation in plasma membranes

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The (Ca^{2+} + Mg^{2+})-ATPase from erythrocyte ghosts catalyzed the hydrolysis of ATP together with the synthesis of ATP or ATP ⇋ P_i exchange. The modulation of the ATPase reaction cycle was controlled by high- and low-affinity calcium-binding sites asymmetrically located on the enzyme. Calmodulin accelerated the reaction cycle in both directions, stimulating the overall turnover of the enzyme. Calcium transport was achieved utilizing optimal conditions for the expression of the ATP ⇋ P_i exchange system.

Introduction

As shown by the number of reports published in the last few years, the plasma membrane (Ca^{2+} + Mg^{2+})-ATPase has been demonstrated to be an important molecule whereby excitable [1–5] and nonexcitable cells [6,7] couple the hydrolysis of ATP to the extrusion of calcium ions from the cytoplasm to the extracellular space. Although the hydrolytic component of the reaction has been extensively studied in different cells types [1–9], the reversal of the ATP-driven calcium pump has not been investigated in plasma membranes as deeply as in cytoplasmic membranes [10–13]. The reversal of the ATP-driven calcium pump or ATP ⇋ P_i exchange mechanism, first described for the sarcoplasmic reticulum [13], has been extensively studied by De Meis and his group since 1972. They incorporated a complete sequence of reactions that can either flow in the direction of the ATP hydrolysis or in the opposite way in the direction of the synthesis of ATP [14–15].

Bond and Clough [16] were first to report that the plasma membrane (Ca^{2+} + Mg^{2+})-ATPase activity might be regulated by a soluble protein, lately known to correspond to calmodulin [17]. This calcium-binding protein shown to stimulate the plasma membrane (Ca^{2+} + Mg^{2+})-ATPase in most cell types by increasing the enzyme affinity for calcium and the V_{max} of the reaction [6,18–19], nowadays has been recognized as an important regulatory protein of the plasma membrane calcium pump. Although the calmodulin effect has been traditionally studied only upon the hydrolytic sequence of reactions, it has been recently suggested that calmodulin might also enhance the reverse reaction or ATP ⇋ P_i exchange, apparently stimulating the overall reaction sequence of the plasma membrane (Ca^{2+} + Mg^{2+})-ATPase [12].

During the present investigation, in order to find out if the calmodulin-sensitive ATP ⇋ P_i exchange mechanism carried out by the sarcolemmal (Ca^{2+} + Mg^{2+})-ATPase is a general feature of other plasma membrane (Ca^{2+} + Mg^{2+})-ATPases, the ATP ⇋ P_i exchange mechanism was studied in erythrocyte ghosts. The finding of this exchange mechanism or ATP synthesis in the system might contribute to the understanding of the concept of

energy conservation at the level of plasma membranes.

Materials and Methods

Vesicle characterization. Since the red blood cells have frequently been employed in the study of the plasma membrane (Ca^{2+} + Mg^{2+})-ATPase and the effect of calmodulin upon this activity, during the present investigation employing a preparation of inside-out ghosts, the regulatory properties of calmodulin upon the overall turnover of the enzyme were studied. Rabbit erythrocyte ghosts were prepared employing several modifications to the method described by Waisman et al. [17]. In order to separate the inside-out from the right-side-out vesicles, a wheat germ agglutinin Sepharose 6MB column was employed as previously described by us [20]. After the vesiculation procedure was completed, 3 ml vesicles resuspended in a buffer containing 0.5 mM sodium phosphate (pH 8.0) were eluted through 2 ml wheat germ agglutinin Sepharose 6MB (Pharmacia). The eluate was collected and diluted with 10 vol. 40 mM glycylglycine (pH 7.1) and centrifugated at 35 000 × *g*. The resulting pellet was resuspended in 4 ml glycylglycine buffer and the vesicles were utilized during the same day. The calmodulin-free vesicles were obtained following the method developed by Caroni [19] and the protein concentration was determined by an adaptation of the method described by Bradford [21].

Kinetic measurements. ATP hydrolysis was measured as release of [^{32}P]P_i from [γ-^{32}P]ATP (3000 Ci/mmol), and ATP synthesis was followed as ATP ⇋ P_i exchange by determining the incorporation of [^{32}P]P_i (40 mCi/ml) into ATP in the presence of ADP. [^{32}P]P_i was extracted from the reaction mixture into an organic phase (*n*-butylacetate) as phosphomolybdate, employing the method previously described by De Meis and co-workers [22]. Samples from the organic and aqueous phases were withdrawn for determination of [^{32}P]P_i and [γ-^{32}P]ATP by scintillation spectrometry. The free calcium concentrations were calculated using a computer program and available stability constants [23].

Calcium transport. Calcium transport by the erythrocyte ghosts was studied using the Millipore Filtration Method. Membrane protein (50–100 μg) was incubated in the same medium utilized for the measurement of the ATP hydrolytic and ATP synthetic reactions with the only extra addition of ^{45}Ca (10 mCi/mg Ca^{2+}). The calcium-transport reaction was initiated with the addition of the membrane vesicles and terminated at the indicated time intervals by filtering aliquots through Millipore filters (HA 0.45 μm) under suction. The filters were washed with 30 ml 50 mM Tris-maleate buffer (pH 7.4), 100 μM $CaCl_2$ and dried at room temperature in scintillation vials before the addition of 5 ml tritosol. The vials were counted in a liquid-scintillation spectrometer.

Results and Discussion

As shown in Table I, before the elution of the ghost sample through the lectin column, the addition of Triton X-100 to this preparation unmasked 30% of the (Ca^{2+} + Mg^{2+})-ATPase activity not measurable during control conditions without the detergent addition. This result confirmed the presence of a mixed type vesicle population in the original ghost preparation. However, when the vesicle preparation was eluted through the lectin column, although 40% of the total protein which corresponded to the right-side-out vesicles was kept bound to the Sepharose column, the eluted vesicles showed a similar (Ca^{2+} + Mg^{2+})-ATPase activity

TABLE I

DETERMINATION OF VESICLE SIDEDNESS

Mixed-type vesicles (before elution)			Inside-out vesicles (after elution)		
Initial protein (mg)	(Ca^{2+} + Mg^{2+})-ATPase activity (nmol/mg per min)		Protein recovered (mg)	(Ca^{2+} + Mg^{2+})-ATPase activity (nmol/mg per min)	
	− Triton X-100	+ Triton X-100		− Triton X-100	+ Triton X-100
20.0	18.0 ± 1.1	25.7 ± 2.4	12.0 ± 3.7	22.6 ± 1.8	23.0 ± 1.9

whether or not detergent was present in the incubation medium (Table I). This result suggested that the eluted fraction was mainly composed of inside-out vesicles with the catalytic site of the enzyme facing the external surface of these vesicles.

In accordance with other authors [24,25] the basal (Ca^{2+} + Mg^{2+})-ATPase activity found in the calmodulin-depleted inside-out erythrocyte vesicles was dependent upon the external free calcium concentration of the reaction mixture (Fig. 1). The (Ca^{2+} + Mg^{2+})-ATPase activity was found to be progressively inhibited when the calcium concentration of the medium was raised from 1 to 5 mM (Fig. 1). Although no ATP ⇋ P_i exchange was detectable at low calcium concentrations, when the concentration of this cation in the medium was progressively raised in the presence of constant P_i and ADP concentrations, an activation of the ATP ⇋ P_i exchange reaction was observed, reaching the plateau at the calcium concentration range of 4–5 mM (Fig. 1). The addition of bovine brain calmodulin to the calmodulin-depleted vesicles, increased the affinity of the ATPase for calcium and stimulated the V_{max} of the reaction (Fig. 1). Moreover, when these vesicles were replenished with the activator protein, calmodulin, the calcium-dependent ATP ⇋ P_i exchange reaction was also enhanced (Fig. 1).

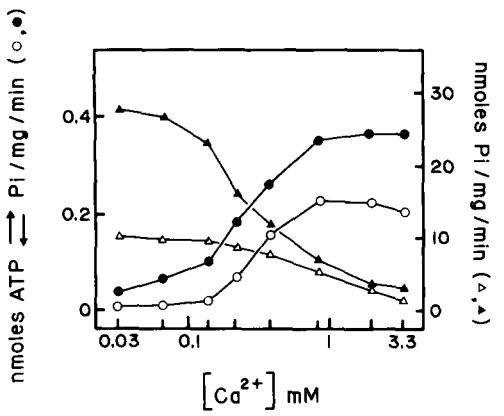

Fig. 1. Effect of calcium on the rate of ATP hydrolysis and ATP ⇋ P_i exchange. ATP hydrolysis (△, ▲): The assay medium contained 50 mM Tris-maleate buffer (pH 7.4), 10 mM $MgCl_2$, 4 mM P_i, 0.2 mM ADP, 6 mM ATP, 10 μM ouabain, 0.01% Triton X-100 and the necessary $CaCl_2$ additions in order to give the free calcium concentrations indicated in the figure. ATP ⇋ P_i exchange (○, ●): Same conditions as for the hydrolysis reaction except that nonradioactive ATP and [^{32}P]P_i were utilized. Calmodulin-free vesicles (△, ○) and vesicles with 1 μg calmodulin/ml added (▲, ●) were preincubated at 4°C for 3 h in the presence of 0.01% Triton X-100 before the initiation of the assay, which was performed for 5 min at 37°C. Representative experiment.

Fig. 2 shows the ATP dependence of the (Ca^{2+} + Mg^{2+})-ATPase activity studied with calmodulin-free vesicles and vesicles with exogenous calmodulin. Under both conditions, it was observed that optimal enzyme activity was achieved when the ATP concentration of the medium was progressively increased. In accordance with other reports [25], it seems that the presence of calmodulin bound to the enzyme enhances the efficiency of the system for the utilization of ATP (Fig. 2). In contrast, if optimal conditions for the measurement of ATP ⇋ P_i exchange were again used employing depleted and calmodulin-repleted vesicles, an increase in the ATP concentration of the medium slightly decreased the capacity of the enzyme to reverse the reaction. Under these conditions, calmodulin seemed to interact with the enzyme in a manner which optimized the ATP ⇋ P_i exchange at low ATP concentrations. These results suggested that the presence of ATP in the medium might contribute to the modulation of the ATP ⇋ P_i exchange process in this cell type. Since these

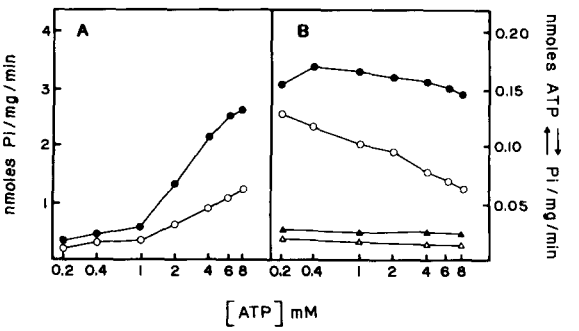

Fig. 2. Effect of ATP on the rates of ATP hydrolysis and ATP ⇋ P_i exchange. (A) ATP hydrolysis: the assay medium contained 50 mM Tris-maleate buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM ADP, 4 mM P_i, 10 μM ouabain, 0.01% Triton X-100 and 0.2, 0.4, 1.0, 2.0, 4.0, 5.0 or 8.0 mM ATP. (B) ATP ⇋ P_i exchange: Same conditions as described in (A) except that nonradioactive ATP and [^{32}P]P_i were used. Calmodulin-free vesicles (○) and vesicles added with 1 μg exogenous calmodulin/ml (●) were treated as explained in Fig. 1. Vesicles were incubated in the absence of detergent, minus calmodulin (△) and plus calmodulin (▲). Representative experiment.

experiments were performed in the presence of Triton X-100 in the incubation medium, an interesting observation was that no ATP ⇆ P_i exchange was detected when the detergent was omitted from the incubation medium and therefore the vesicles remained intact (Fig. 2). It was observed that no ATP ⇆ P_i exchange developed probably due to the short incubation period and the impossibility of the calcium pump to build up the necessary calcium concentration inside the vesicle in order to saturate the low-affinity sites and switch on the ATP ⇆ P_i exchange reaction. This result is consistent with the observation made for the sarcoplasmic reticulum and the red cell [24–26], which suggests the presence of a low-affinity site for calcium not associated with the catalytic unit of the enzyme located at the inner surface of the inside-out ghosts that is needed for the expression of the ATP ⇆ P_i exchange reaction or synthesis of ATP.

When incubated in a medium comprising of 50 mM Tris-maleate buffer (pH 7.4)/100 μM $CaCl_2$/1 mM $MgCl_2$/4 mM P_i/0.2 mM ADP/6 mM ATP/10 μM ouabain, the inside-out ghosts were able to catalyze the accumulation of calcium while hydrolyzing [^{32}P]ATP or concomitantly synthetizing this nucleotide from ADP and [^{32}P]P_i (Fig. 3). From these experiments, it is interesting to point out that although the (Ca^{2+} + Mg^{2+})-ATPase activity slowed down after 15 min of incubation, the rate of the ATP ⇆ P_i exchange reaction and calcium transport continued at the same original rate. This is consistent with the fact that calcium might be accumulating and binding to the low-affinity site of the enzyme located at the inner surface of the membrane, allowing the activation of the exchange reaction. Although there is evidence indicating that a small fraction of the calcium influx is associated with the Ca^{2+}-pump reversal [27], the results reported here suggest that independently of the formation of a transmembrane calcium gradient, the binding of calcium and ATP to the enzyme modulate the rates of both the hydrolysis and the synthesis of ATP. Moreover, in accordance with an earlier observation [12], this study supports the idea of calmodulin being an activator of the overall turnover of the plasma membrane (Ca^{2+} + Mg^{2+})-ATPase.

Although in intact cells the catalytic unit of the enzyme associated with the high-affinity site for calcium is located at the cytoplasmic side of the membrane, the low-affinity site for this cation is thought to be integrated outside the membrane in direct contact with a constant high extracellular calcium concentration. It might be therefore suggested that under normal conditions, the extrusion

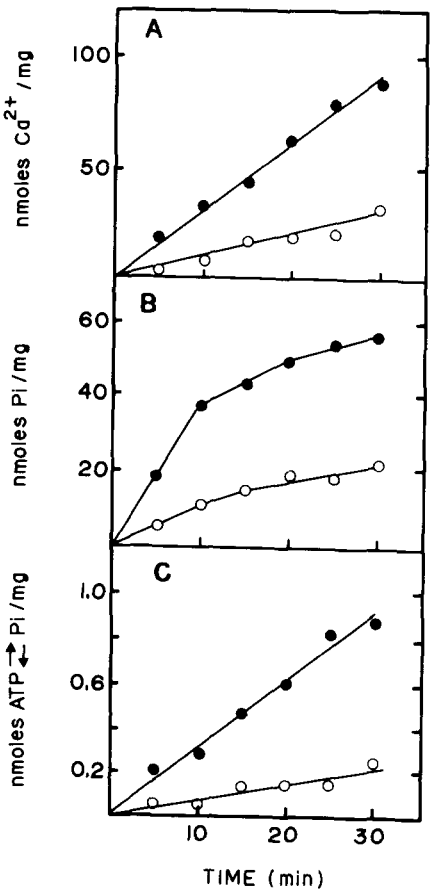

Fig. 3. Stimulation of the (Ca^{2+} + Mg^{2+})-ATPase activity, ATP ⇆ P_i exchange and calcium transport by calmodulin utilizing intact inside-out erythrocyte ghosts. The assay medium contained 50 mM Tris-maleate buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM ATP, 0.2 mM ADP, 4 mM P_i, 0.1 mM $CaCl_2$ and 10 μM ouabain. Minus calmodulin (○); plus 1 μg calmodulin/ml (●). (A) Calcium-transport assay performed using nonlabelled ATP, P_i and $^{45}CaCl_2$. (B) ATP hydrolysis assay carried out using nonlabelled P_i, $CaCl_2$ and [γ-^{32}P]ATP. (C) ATP ⇆ P_i exchange reaction performed employing nonlabelled ATP, $CaCl_2$ and [^{32}P]P_i. Samples were incubated at 37°C, withdrawn at the indicated times and either treated for the determination of [^{32}P]P_i and [γ-^{32}P]ATP or filtered through Millipore filters for the determination by scintillation spectrometry of the ^{45}Ca accumulated in the vesicles.

of calcium from the cytoplasm of the erythrocyte to the extracellular space is indeed supported by the hydrolytic reaction of the $(Ca^{2+} + Mg^{2+})$-ATPase working under favorable conditions for the expression of the reverse reaction of the cycle and the synthesis of ATP.

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