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EXCHANGE BETWEEN INORGANIC PHOSPHATE AND ADENOSINE TRIPHOSPHATE IN (Na^+, K^+)-ATPase

VERA LUCIA GONÇALVES DE MORAES and LEOPOLDO DE MEIS

Instituto de Ciências Biomédicas, Departamento de Bioquímica, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Cidade Universitária, Ilha do Fundão, 21.910, Rio de Janeiro, RJ (Brasil)

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(Na^+, K^+)-ATPase is able to catalyze a continuous ATP $\rightleftharpoons$ P_i exchange in the presence of Na^+ and in the absence of a transmembrane ionic gradient. At pH 7.6 the Na^+ concentration required for half-maximal activity is 85 mM and at pH 5.1 it is 340 mM. In the presence of optimal Na^+ concentration, the rate of exchange is maximal at pH 6.0 and varies with ADP and P_i concentration in the assay medium. ATP $\rightleftharpoons$ P_i exchange is inhibited by K^+ and by ouabain.

Introduction

The (Ca^{2+}, Mg^{2+})-dependent ATPase of sarcoplasmic reticulum and the (Na^+, K^+)-ATPase from plasma membrane are able to catalyze the hydrolysis and the synthesis of ATP [1–6]. Synthesis of ATP is observed when a gradient of either Ca^{2+} or Na^+ is formed across the membrane.

Under appropriate conditions, the (Ca^{2+}, Mg^{2+})-dependent ATPase is able to catalyze an ATP $\rightleftharpoons$ P_i exchange reaction. During this reaction the enzyme operates simultaneously forward, in the direction of ATP hydrolysis, and backward, in the direction of ATP synthesis. In early reports it was proposed that the energy for the synthesis of ATP, measured during the exchange reaction, was derived from the Ca^{2+} gradient formed across vesicle membranes [4]. Later, it was demonstrated that the (Ca^{2+}, Mg^{2+})-dependent ATPase was able to catalyze a continuous ATP $\rightleftharpoons$ P_i exchange, in the absence of transmembrane gradient [7–9]. Using the (Na^+, K^+)-ATPase, Post and coworkers [10,11] were able to measure ATP synthesis and ATP $\rightleftharpoons$ P_i exchange, after a single catalytic cycle of the enzyme, in the absence of an Na^+ gradient. This was achieved after a sequential addition of reactants to a medium containing the enzyme.

In this report it is shown that (Na^+, K^+)-ATPase, like the (Ca^{2+}, Mg^{2+})-dependent ATPase, is able to catalyze a continuous ATP $\rightleftharpoons$ P_i exchange, under steady-state conditions, in the absence of an ionic transmembrane gradient.

Material and Methods

The (Na^+, K^+)-ATPase was purified from outer medulla of sheep kidney as described by Jørgensen [12]. The specific activity was found to vary between 3 and 5 μmol P_i/min per mg protein. This was measured at 37°C using a medium containing 3 mM Mg^{2+}, 3 mM ATP, 130 mM Na^+ and 20 mM K^+, at pH 7.4. Ouabain-insensitive ATPase activity was not detectable.

[γ-^{32}P]ATP was prepared as previously described [13].

$^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy and was purified by extraction as phosphomolybdate with a mixture of benzene and isobutyl alcohol, reextraction to the aqueous phase

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with ammonium hydroxide solution, and finally precipitation as the $MgNH_4PO_4$ salt [14]. The $^{32}P_i$ was stored in dilute HCl solution until used.

$ATP \rightleftharpoons P_i$ exchange was assayed by measuring the formation of [γ-^{32}P]ATP from ADP and $^{32}P_i$. The excess of $^{32}P_i$ was removed from the medium as phosphomolybdate with isobutyl alcohol-benzene [7,15]. In each experiment the reaction was arrested by mixing 0.2 ml of the assay medium with 0.05 ml of a trichloroacetic acid 50% (w/v) solution. This was followed by the addition of 0.3 ml of acetone, 0.4 ml of 1.25 M H_2SO_4 solution containing 5% (w/v) ammonium molybdate and 1 ml of a mixture of isobutyl alcohol and benzene (v/v), saturated with water. The tubes were vigorously stirred for 1 min in a Vortex. After phase separation, the isobutyl alcohol-benzene layer was removed; 0.01 ml of 20 mM KH_2PO_4 was added to the aqueous layer and the mixture was extracted with isobutyl alcohol-benzene. This step was repeated twice. An aliquot of the water phase was counted in a liquid scintillation counter. In this extraction only 0.01–0.05% of the original content of $^{32}P_i$ remained in the aqueous phase. This was negligible in the standard assay.

ATPase activity was assayed by measuring the release of $^{32}P_i$ from [γ-^{32}P]ATP. The $^{32}P_i$ was extracted from the assay medium as described above, except that after the first extraction with isobutyl alcohol-benzene the tube was centrifuged for 3 min at $5000 \times g$ and an aliquot of the isobutyl alcohol-benzene phase was counted.

Protein was assayed according to the method of Lowry et al. [16], using bovine serum albumin as standard.

Reagents. Vanadate free ATP and ADP were obtained from Boehringer Mannheim. In all experiments the sodium salt of ATP, ADP and orthophosphate were used. Inorganic salts and other reagents were purchased from either Carlos Erba or E. Merck, Darmstadt. Stock solutions of radioactive phosphate contained 20 to 50 mM NH_4Cl derived from purification procedure. In the different experiments stock solutions were diluted 25- to 80-fold. Thus, the final contaminant NH_4Cl concentration in the assay medium was 0.25 to 2 mM. In the presence of optimum Na^+ concentrations the rate of $ATP \rightleftharpoons P_i$ exchange was not modified by the addition of 2 to 20 mM NH_4Cl in the assay medium.

High pressure liquid chromatography. The radioactive ATP formed during $ATP \rightleftharpoons P_i$ exchange reaction was identified by using Waters equipment. Samples were eluted in a C-18 column (2 ml/min) with 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) at 25°C. In a typical experiment the reaction was quenched by HCl to a final concentration of 1 M. Excess of $^{32}P_i$ was removed from the medium as phosphomolybdate with isobutyl alcohol-benzene. This was followed by two additions of KH_2PO_4 and reextracted with isobutyl alcohol-benzene. The remaining molybdate was removed from the medium by adding excess of KH_2PO_4 (50 mM) and reextracted as above. The water phase obtained was adjusted to pH 6.0 with 4 M NaOH and passed through a 0.45 μm Millipore filter. An aliquot of 0.2 ml was injected in the column. Samples of 1 ml were collected and used for measuring both absorbance at 254 nm and radioactivity after extraction with isobutyl alcohol-benzene.

Results

Na^+-ATPase activity and $ATP \rightleftharpoons P_i$ exchange

ATP hydrolysis and $ATP \rightleftharpoons P_i$ exchange rates were measured at pH 7.40, in the presence of increasing Na^+ concentrations and no added K^+ (Fig. 1). Little or no $ATP \rightleftharpoons P_i$ exchange was measured in a range of 1 to 25 mM NaCl. However a progressive activation of $ATP \rightleftharpoons P_i$ exchange was observed when Na^+ concentration of the medium was raised, reaching a maximum at about 200 mM NaCl.

In the presence of 200 to 300 mM NaCl, the rate of ATP hydrolysis was found to be 40- to 120-times faster than the rate of $ATP \rightleftharpoons P_i$ exchange (Fig. 1, Table I). This means that in the presence of an optimal NaCl concentration, 40 to 120 molecules of ATP are hydrolysed for every molecule of ATP synthesized from ADP and P_i. A similar ratio was previously reported from the $ATP \rightleftharpoons P_i$ exchange catalyzed by the (Ca^{2+}, Mg^{2+})-dependent ATPase of sarcoplasmic reticulum, in the absence of Ca^{2+} gradient [7–9]. Addition of NaCl in excess (1 M) leads to an inhibition of $ATP \rightleftharpoons P_i$ exchange reaction. ATPase activity did not vary significantly in the range of 25 to 1000 mM NaCl (Fig. 1).

In the presence of optimal Na^+ concentration,

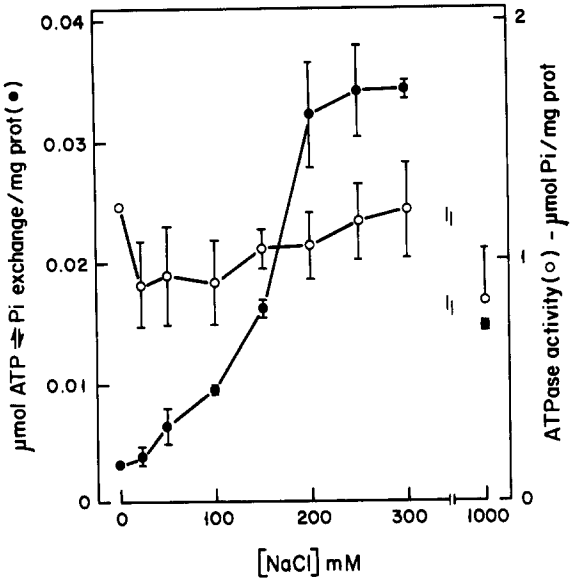


Fig. 1. Na^+-ATPase activity and ATP⇌P_i exchange. The assay medium composition was 50 mM Tris-maleate buffer (pH 7.4), 1 mM ATP, 0.2 mM ADP, 15 mM $MgCl_2$, 10 mM P_i and 25 μg of (Na^+, K^+)-ATPase protein. The final volume was 0.2 ml. For the ATPase activity (○), [γ-^{32}P]ATP and non-radioactive P_i were used. For ATP⇌P_i exchange (●), $^{32}P_i$ and non-radioactive ATP were used. The reaction was started by the addition of the enzyme and stopped with 0.05 ml trichloroacetic acid 50% (w/v), after 15 min at 36.5°C. The values shown are the average±S.E. of three experiments.

the amount of $^{32}P_i$ incorporated into the ATP pool increased linearly as a function of incubation time (Fig. 2A). Minimum molecular weight of (Na^+, K^+)-ATPase is in the range of 270000 to 286000 [17]. Under the conditions of Fig. 2, after 20 min of incubation each 3.5 nmol of enzyme (1 mg of protein) did catalyze the synthesis of 50 nmol of ATP.

These data show that during steady state of ATP hydrolysis, each enzyme unit is able to catalyze the synthesis of several ATP molecules.

Na^+ requirement for the ATP⇌P_i exchange reaction was found to vary depending on the pH of the medium. NaCl concentrations required for half-maximal rate of ATP⇌P_i exchange at pH 5.1, 5.9, 7.0 and 7.6 were found to be 340, 240, 220 and 85 mM NaCl, respectively. At all pH values tested, addition of excess NaCl to the medium lead to an inhibition of ATP⇌P_i exchange reaction (Fig. 3A). In the presence of optimal NaCl con-

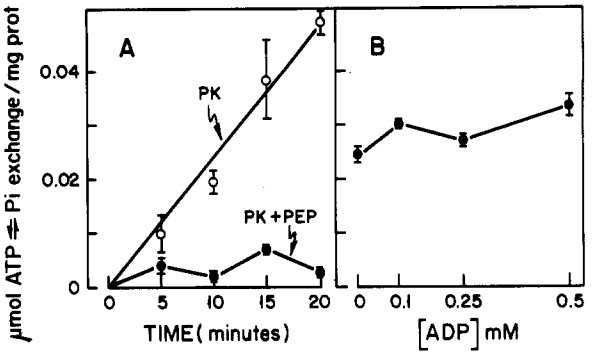


Fig. 2. Incubation time and ADP dependence. (A) The assay medium composition was 50 mM Tris-maleate buffer (pH 7.5), 1 mM ATP, 15 mM $MgCl_2$, 400 mM NaCl, 12 mM P_i, $^{32}P_i$, 18 μg of (Na^+, K^+)-ATPase protein and either 8 μg/ml pyruvate kinase alone (○) or 8 μg pyruvate kinase plus 2.5 mM phospho*enol*pyruvate (●). The final volume was 0.2 ml. The values represent the average±S.E. of three experiments. (B) In a final volume of 0.2 ml, the reaction mixture contained 25 μg of (Na^+, K^+)-ATPase protein; 8 mM Tris-maleate buffer (pH 7.5); 380 mM NaCl, 23 mM $MgCl_2$, 1.5 mM ATP and 15 mM P_i. Each value is the average of two determinations. Both in (A) and in (B) the reaction was started by the addition of the enzyme and stopped with 0.05 ml trichloroacetic acid 50% (w/v), after 15 min at 36°C.

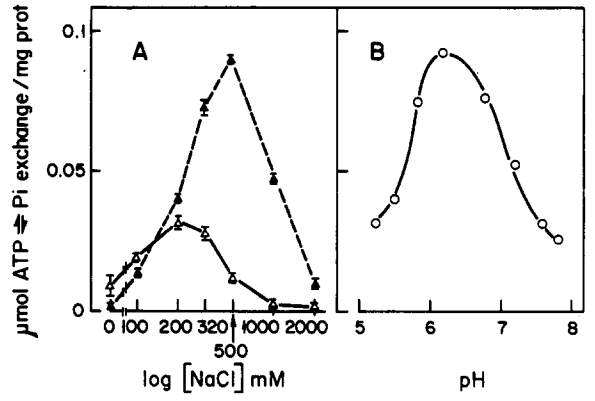


Fig. 3. (A) Effect of pH on Na^+ requirement of ATP⇌P_i exchange. ▲, pH 5.9; △, pH 7.64. (B) Maximal rate of ATP⇌P_i exchange in the presence of optimal NaCl concentrations: 500 mM for pH 5.2 to pH 6.8; 300 mM for pH 7.2 and 200 mM for pH 7.64 and 7.8. For both figures the assay medium composition was 50 mM Tris-maleate buffer, 1 mM ATP, 0.2 mM ADP, 15 mM $MgCl_2$, 10 mM P_i. The final volume was 0.2 ml and contained 18 μg of (Na^+, K^+)-ATPase protein. The final pH of mixture was adjusted to the desired value by adding Tris base or maleic acid. The reaction started with addition of enzyme and was stopped with 0.05 ml trichloroacetic acid 50% after 15 min at 36°C. The values are the average±S.E. of three experiments.

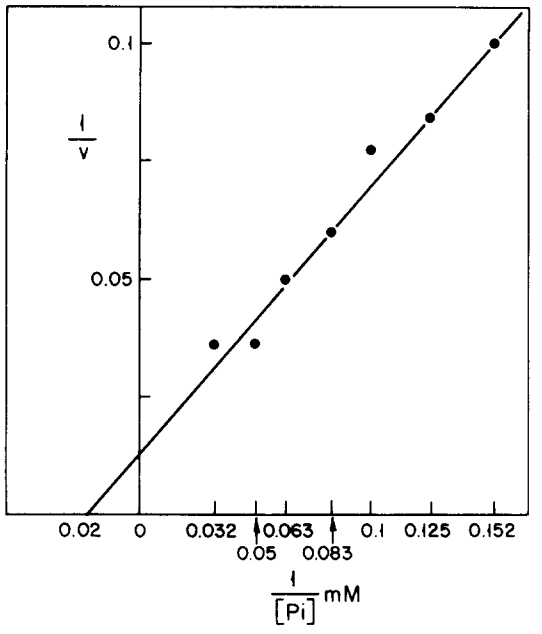


Fig. 4. P_i dependence. In a final volume of 0.2 ml, the reaction mixture contained 18 μg of (Na^+, K^+)-ATPase protein, 50 mM Tris-maleate buffer, 400 mM NaCl, 15 mM $MgCl_2$, 1 mM ATP, 0.2 mM ADP and 6.6, 8.0, 10, 12, 16, 20 and 32 mM $^{32}P_i$. The final pH of the mixture was 7.0. The reaction started with addition of enzyme and was stopped with 0.05 ml trichloroacetic acid 50% after 15 min at 36.5°C. The values shown are the average ± S.E. of three experiments.

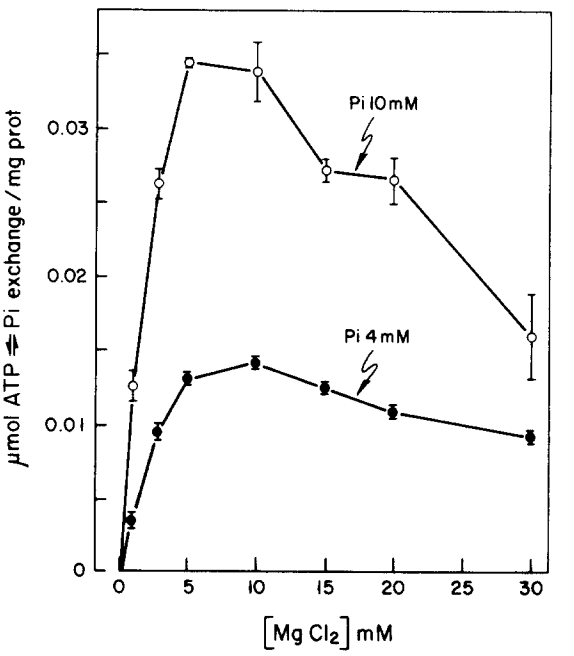


Fig. 5. Mg^{2+} dependence. The assay medium composition was 50 mM Tris-maleate buffer (pH 7.4), 1 mM ATP, 0.2 mM ADP, 400 mM NaCl and 18 μg of (Na^+, K^+)-ATPase protein. The final volume was 0.2 ml. The P_i concentration added was 4 mM (●) or 10 mM (○). The reaction was started by the addition of the enzyme and stopped with 0.05 ml trichloroacetic acid 50%, after 15 min at 37°C. The values shown are the average ± S.E. of three experiments.

centrations, maximal rate of exchange also varied with the pH of the assay medium (Fig. 3B). Maximal rate was found to be at about pH 6.0. In contrast the optimal pH for ATPase activity dependent on Na^+ and K^+ is in the range of pH 7.0–7.4 [18].

ADP dependence

ADP is required as a substrate for the ATP ⇌ P_i exchange (Fig. 2). The formation of [γ-^{32}P]ATP was arrested if an ATP regenerating system was included in the assay medium in order to avoid the accumulation of ADP derived from the ATPase activity (Fig. 2A). Addition of increasing concentrations of ADP to the assay medium did not change significantly the rate of ATP ⇌ P_i exchange (Fig. 2B). These data show that the ADP derived from the ATPase activity was sufficient for maximal activation of the rate of [γ-^{32}P]ATP formation. Therefore we could not measure the apparent K_m of ADP for this reaction.

P_i dependence

The rate of ATP ⇌ P_i exchange varies with the P_i concentration of the medium (Fig. 4). In four experiments the apparent K_m for P_i was found to vary between 40 and 50 mM. A similar apparent K_m for P_i was previously reported for ATP ⇌ P_i exchange catalyzed by sarcoplasmic reticulum ATPase, in the absence of a Ca^{2+} gradient [7,8].

Mg^{2+} dependence

Magnesium is required as an activating ion for the ATP ⇌ P_i exchange reaction. No detectable ATP ⇌ P_i exchange was measured in the absence of Mg^{2+}. Under conditions of Fig. 5, maximal activation was attained in the presence of 5 to 10 mM $MgCl_2$. Excess of magnesium was inhibitory.

ATP dependence

The same rate of ATP ⇌ P_i was measured in the presence of either 1 or 10 mM ATP (data not

shown). This was measured using a medium containing 0.2 mM ADP, 400 mM NaCl, 15 mM $MgCl_2$ and 12 mM P_i, at pH 7.50.

Inhibition by K^+ and ouabain

$ATP \rightleftharpoons P_i$ exchange reaction was inhibited when 20 mM K^+ was added to the medium (Table I). This was accompanied by a substantial activation of the ATPase activity. Ouabain is a specific inhibitor of (Na^+, K^+)-ATPase. Both the ATPase activity and the $ATP \rightleftharpoons P_i$ exchange reaction were found to be inhibited after the addition of 1 mM ouabain to the assay medium (Table I).

$ATP \rightleftharpoons P_i$ was not inhibited by 20 mM NH_4Cl. Higher concentrations of NH_4Cl were not tested (data not shown).

Identification of [γ-^{32}P]ATP

The experiment described in Fig. 6 was performed to show that the radioactivity measured after extraction with isobutyl alcohol-benzene was in fact ATP. The figure shows that a peak of radioactivity and of ultraviolet light absorbance was attained in the same samples where ATP should be eluted. The radioactivity peak was abolished when the ATP contained in the assay medium was hydrolyzed after prolonged incubation with an excess of (Na^+, K^+)-ATPase.

TABLE I

INHIBITION BY K^+ AND OUABAIN

The assay medium contained 50 mM Tris-maleate buffer (pH 7.5), 2 mM ATP, 0.2 mM ADP, 5 mM $MgCl_2$, 12 mM P_i and 18 μg of ATPase. The final volume was 0.2 ml. The time of incubation was 15 min. The values of $ATP \rightleftharpoons P_i$ exchange, measured in absence and presence of K^+, are the average ± S.E. of ten different experiments. The remaining data are the average of duplicate experiments.

Addition	ATPase activity (μmol P_i/mg protein)	$ATP \rightleftharpoons P_i$ exchange (nmol/mg protein)
200 mM NaCl	4.3	34.7 ± 2.1
200 mM NaCl plus 200 mM KCl	25.0	1.6 ± 0.7
400 mM NaCl plus 1 mM ouabain	0	0.8

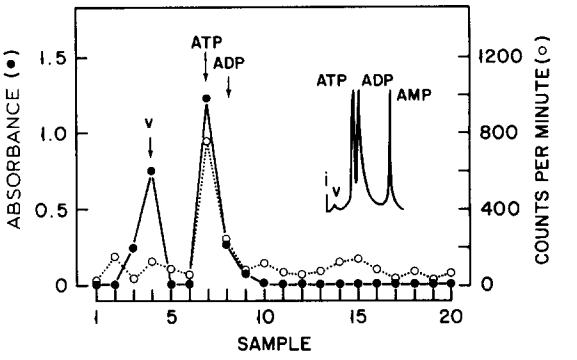
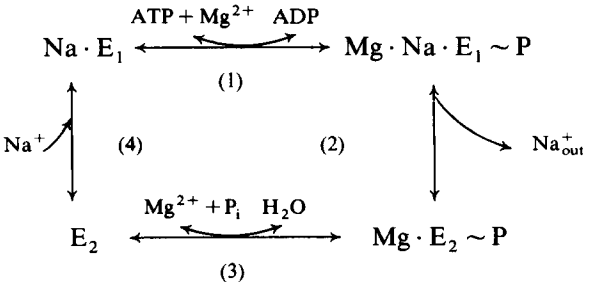


Fig. 6. Identification of [γ-^{32}P]ATP. $ATP \rightleftharpoons P_i$ exchange reaction was assayed in a 2 ml medium containing 400 mM NaCl, 15 mM $MgCl_2$, 12 mM $^{32}P_i$, 0.2 mM ADP, 10 mM ATP, 50 mM Tris-maleate buffer and 50 μg of protein/ml. The final pH was 7.06 at 25°C. The reaction started with addition of the enzyme and was stopped with 0.4 ml 6 M HCl after 45 min at 36°C. The preparation of the sample and conditions for chromatography are described under Methods. The inset shows an elution pattern of control mixture containing equimolar concentrations of ATP, ADP and AMP. In the figure *v* refers to void volume and *i* to injection of the sample into the column. ●, absorbance at 254 nm; ○, radioactivity. No radioactivity was detected after isobutyl alcohol-benzene extraction, when the reaction was performed in the same conditions as described above, except that the ATP concentration was decreased to 1 mM and the (Na^+, K^+)-ATPase increased to 300 μg/ml. After 45 min all ATP of the medium was hydrolyzed.

Discussion

(Na^+, K^+)-ATPase prepared as described by Jørgensen [19] is obtained as loose sheets of membrane not sealed as vesicles. Therefore, the $ATP \rightleftharpoons P_i$ exchange measured in Figs. 1 to 5 was catalyzed by the enzyme in the absence of an Na^+ gradient across the membrane.

$ATP \rightleftharpoons P_i$ exchange reaction can be better discussed on the basis of the following sequence:



This sequence is the same as that proposed by Taniguchi and Post [11], with the exception that K^+ was omitted from the system. E_1 and E_2 represent two distinct functional states of the enzyme. The enzyme from E_1 has a high affinity for Na^+ and is phosphorylated by ATP, but not by P_i. The enzyme form E_2 has a lower affinity for Na^+ and is phosphorylated by P_i, but not by ATP. Only the phosphoenzyme form $Mg \cdot Na \cdot E_1 \sim P$ is able to transfer its phosphate to ADP.

According to this sequence, $ATP \rightleftharpoons P_i$ exchange is due to reversal of reactions 1, 2 and 3. The catalytic cycle would be initiated by phosphorylation of $Na \cdot E_1$ by non-radioactive ATP (reaction 1). After reactions 2 and 3, non-radioactive P_i is released in the medium. Reversal of reaction 3 would lead to the formation of radioactive phosphoenzyme formed from $^{32}P_i$, present in the assay medium and reversal of reactions 2 and 1 would lead to the formation of radioactive ATP. High Na^+ concentrations would be required to permit the reversal of reaction 2. The ratio between the amount of ATP hydrolyzed and the amount of ATP synthesized from ADP and P_i would depend on the rate constant forward and backward of reactions 1 to 4. Potassium ion would speed the rate of reaction 3 and 4 forward. As a result ATP hydrolysis is accelerated and ATP synthesis is impaired (Table I). Taniguchi and Post [11] first showed that (Na^+, K^+)-ATPase is able to catalyze an $ATP \rightleftharpoons P_i$ exchange. The enzyme was incubated with 160 mM Na^+, $^{32}P_i$, Mg^{2+}, ADP and non-radioactive ATP. Following this procedure it was found that the enzyme is able to catalyze the formation of [γ-^{32}P]ATP. In these experiments the number of [γ-^{32}P]ATP molecules formed did not exceed the amount of enzyme molecules added (Table VI and Fig. 9 of Ref. 11). However, from the data reported (Fig. 9 of Ref. 11), it could be predicted that the (Na^+, K^+)-ATPase, similarly to the (Ca^{2+}, Mg^{2+})-dependent ATPase of sarcoplasmic reticulum [7–9], should catalyze a continuous $ATP \rightleftharpoons P_i$ exchange, in the absence of a transmembrane ionic gradient. Our results show that the number of [γ-^{32}P]ATP molecules formed from ADP and $^{32}P_i$, exceeds many-fold the number of enzyme molecules added. This can be seen in Figs. 1–5. In accordance with Taniguchi and Post [11] the exchange is inhibited by K^+ and ouabain (Table I). In addition, this report shows that for the $ATP \rightleftharpoons P_i$ exchange reaction: (a) the apparent affinity of the enzyme for ADP is very high (reaction 1) and for P_i is very low (reaction 2, Figs. 2 and 4); (b) apparent K values for Na^+ vary with the pH of the assay medium (reaction 3); (c) excess of Na^+ leads to an inhibition of the exchange reaction (Fig. 3A); (d) in the presence of optimal NaCl concentrations, the rate of exchange varies with the pH of the medium (Fig. 3B).

At present we do not know why excess of Na^+ leads to an inhibition of $ATP \rightleftharpoons P_i$ exchange reaction. The effect of pH on the rate of exchange, measured in the presence of optimal concentrations of Na^+, indicates that the backward rate of one or more steps involved in the exchange is faster at pH 6.0 than at pH 7.4.

An intriguing observation is that pH alters by different manners the affinity of the enzyme for Na^+ and the overall rate of $ATP \rightleftharpoons P_i$ exchange. At more alkaline pH values the affinity for Na^+ is increased while the rate of exchange is decreased (Fig. 3).

As proposed for the (Ca^{2+}, Mg^{2+})-dependent ATPase, it is suggested that the (Na^+, K^+)-ATPase, during $ATP \rightleftharpoons P_i$ exchange, is able to conserve some of the energy released from ATP hydrolysis in a form that permits resynthesis of ATP.

Finally, in the literature it has been shown that in absence of K^+ the ATPase activity is inhibited by low Na^+ concentrations. This inhibition is abolished when the Na^+ concentration is raised from 10 to 150 mM [20–23]. We failed to detect this effect (Fig. 1). Perhaps this discrepancy derives from the fact that we always had Na^+ and NH_4^+ contamination present in the assay medium (see Reagents in Methods). In this report the effect of low Na^+ concentrations was not explored further, because our main interest was to measure rates of ATP hydrolysis in the presence of high Na^+ concentrations which activate $ATP \rightleftharpoons P_i$ exchange.

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