J. Membrane Biol. 45, 1-20 (1979)

Conformational Changes of Membrane-Bound (Na^+-K^+)-ATPase as Revealed by Antibody Inhibition

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Received 8 June 1978

Summary. As different structural states of the (Na^+-K^+)-ATPase (EC 3.6.1.3) may lead to a changed reactivity to antibodies, the influence of Na^+, K^+, Mg^{++}, P_i and ATP on the reaction between highly purified (Na^+-K^+)-ATPase and antibodies directed against the membrane-bound enzyme was measured. The antigen antibody reaction was registered by measuring the antibody inhibition of (Na^+-K^+)-ATPase activity.

In the *membrane-bound* but not in the *solubilized* enzyme four different degrees of antibody inhibition were obtained at equilibrium of the antigen antibody reaction if different combinations of Na^+, K^+, Mg^{++} and ATP were present during the incubation with the antibodies. Corresponding to the different degrees of inhibition, different rates of enzyme inhibition were measured. (a) The smallest degree of enzyme inhibition was obtained when (i) only Mg^{++}, (ii) Mg^{++} and Na^+ or (iii) Mg^{++} and K^+ were present during the antigen antibody reaction. (b) The enzyme activity was inhibited more strongly if Na^+, Mg^{++} and ATP were present together. (c) It was inhibited even more if only (i) Na^+, (ii) K^+, (iii) ATP or both (iv) ATP and Na^+, (v) ATP and K^+, (vi) ATP and Mg^{++}, or if (vii) no ATP and activating ions were present. (d) The highest degree of antibody inhibition was obtained if Mg^{++}, ATP and K^+ were present together.

In the presence of Mg^{++} plus ADP and in the presence of Mg^{++} plus the ATP analog adenylyl (β-γ-methylene) diphosphonate, Na^+ and K^+ did not influence the degree of antibody inhibition as they did in the presence of Mg^{++} plus ATP. It was further found that the degree of antibody inhibition in the presence of Mg^{++}, ATP and K^+ was affected by the sequence in which K^+ and ATP were added to the enzyme prior to the addition of the antibodies.

It is suggested that by antibody inhibition different conformations of the (Na^+-K^+)-ATPase could be detected. These conformations may possibly not occur in the solubilized enzyme and therefore do not seem to be necessarily linked to the intermediary steps of the ATP hydrolysis of the enzyme. The structural changes which are induced by Na^+ and K^+ in the presence of Mg^{++} plus ATP are proposed to occur during the Na^+-K^+ transport.

The Na^+, K^+ and Mg^{++}-activated ATPase has been shown to be closely related to Na^+ and K^+ transport across membranes. Many models as to how the (Na^+-K^+)-ATPase may affect Na^+ and K^+ transport have been proposed. Some of

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those involve conformational changes in selected regions of the polypeptide chains of the enzyme or changes in the spatial relationship between the subunits [1, 10, 25]. Results from different studies have been reported, suggesting that the (Na^+-K^+)-ATPase actually obtains different conformational states: Inactivation experiments with ouabain, N-ethylmaleimide, and trypsin have shown that the inhibition pattern of the (Na^+-K^+)-ATPase activity is altered at a variety of different conditions [1, 7, 11, 16, 23, 24]. The alterations of the (Na^+-K^+)-ATPase structure were studied more directly (i) by calorimetric measurements [14], (ii) by the analysis of splitting products after trypsin proteolysis of membrane-bound (Na^+-K^+)-ATPase [11], (iii) by measuring the fluorescence of (Na^+-K^+)-ATPase which was labeled by a fluorescence probe [8], and (iv) by measuring the degree of antibody inhibition of (Na^+-K^+)-ATPase activity [17] and of ouabain binding [18] at some ligand conditions.

It is difficult to compare the results which were obtained with these methods as only some ATP and ligand combinations were tested by each method and as different enzyme preparations were investigated.

To evaluate the functional relevance of the observed structural states, the protein conformations have to be studied in the presence of all the different possible combinations of ATP and cosubstrates and the studies have to be performed in different enzyme preparations. In addition, the structural changes have to be characterized. This can be done if they are evaluated by different methods. Furthermore, the specificity of the effects of ATP and cosubstrates has to be tested.

We therefore tried to register structural states of the membrane-bound (Na^+-K^+)-ATPase in the presence of many ATP and cosubstrate combinations. The enzyme conformations were evaluated by analyzing the splitting products after trypsin digestion, as we shall describe in another report,[1] and by measuring the influence of all possible Na^+, K^+, Mg^{++} and ATP combinations on the degree of antibody inhibition of the membrane-bound enzyme, assuming that different degrees of antibody inhibition reflect different enzyme conformations. We found that the response of membrane-bound (Na^+-K^+)-ATPase to the antibody inhibition was altered if Mg^{++} was bound to the enzyme. It was further observed that this Mg^{++} effect was balanced by the addition of ATP. Nevertheless, the enzyme structure in the presence of Mg^{++} plus ATP does not seem to be identical to that in the absence of ATP and cosubstrates since it was found that the response of membrane-bound (Na^+-K^+)-ATPase to antibody inhibition was only altered by Na^+ or K^+ if Mg^{++} plus ATP were also present. In the absence of Mg^{++} and ATP and in the presence of Mg^{++} or ATP, Na^+ and K^+ did not influence the antibody inhibition of the enzyme.

Materials and Methods

Preparation of (Na^+-K^+)-ATPase

Membrane-bound (Na^+-K^+)-ATPase from the outer medulla of rat kidneys was purified by incubation of a microsomal fraction with sodiumdodecyl sulfate and ATP followed

[1] Koepsell, H. 1978. Conformational changes of membrane-bound (Na^+-K^+)-ATPase as revealed by trypsin digestion (*unpublished*).

by zonal centrifugation and the membrane-bound enzyme was solubilized by Lubrol WX in the presence of ATP [13]. The specific activities were 30–36 and 15–20 μmol ATP split per mg protein per min for the membrane-bound and solubilized enzyme, respectively. The purity of the enzyme as checked by sodium dodecylsulfate gel electrophoresis was about 95%. Adenine nucleotides were removed from the enzyme by gel chromatography in the presence of 50% glycerol and 3 mM $MgCl_2$ [13].

Preparation of Antibodies

For the antibody inhibition studies gammaglobulin was prepared from rabbits which had been immunized with highly purified membrane-bound (Na^+-K^+)-ATPase. The immunization scheme and the purification procedure for the gammaglobulin is described in a previous paper [13]. Here also a characterization of the (Na^+-K^+)-ATPase antibodies which were used in the study is given.

Protein and (Na^+-K^+)-ATPase Assay

Protein was determined according to the method of Lowry and coworkers [15] with bovine serum albumin (BSA) as a standard solution. The (Na^+-K^+)-ATPase activity was assayed by the pyruvate kinase-lactate dehydrogenase linked system in which hydrolysis of ATP is coupled to the oxidation of NADH [20]. Final concentrations were: 70 mM imidazole-HCl, pH 7.3, 5 mM $MgCl_2$, 150 mM NaCl, 100 mM NH_4Cl which was introduced instead of KCl [20], 3 mM ATP (Na_2) obtained from Boehringer (Mannheim), 0.4 mM NADH, 0.6 mM phosphoenolpyruvate, 6 IU/ml pyruvate kinase, 6 IU/ml lactic dehydrogenase. Temperature 37 °C, λ = 340 nm. In order to compensate for the activity of Mg^{++}-stimulated ATPase all readings were taken against a control in which NH_4Cl was omitted and 2.4 mM ouabain was present in the test. The reaction was started by adding the enzyme to the prewarmed reaction mixture, and the initial linear rate of (Na^+-K^+)-ATPase activity was measured within the next two minutes.

Measurements of Antibody Inhibition in the Presence of Different Ligands

To determine the effects of cations and nucleotides on the antibody inhibition, time courses of antibody inhibition of (Na^+-K^+)-ATPase activity and dose response curves in the presence of different ligands were measured. To register the time course of antibody inhibition, the enzyme was first preincubated at 0 °C (30 min) in a buffer containing 20 mM imidazole-HCl, pH 7.5, 2 mM EGTA, 1 mg/ml BSA and, in addition, phosphate and nucleotides as described in *Results.* After the preincubation period, gammaglobulin which had been dialyzed against a buffer containing 20 mM imidazole-HCl, pH 7.5, and 2 mM EGTA was added and mixed at 0 °C. Samples were taken at different time intervals after addition of the antibodies and the initial rate of (Na^+-K^+)-ATPase activity was immediately assayed. Time courses of antibody inhibition in the presence of different ligands were followed, and the degrees of inhibition at equilibrium of antigen antibody reactions were compared, keeping the enzyme and gammaglobulin concentrations constant. As different gammaglobulin and enzyme preparations had to be used, the experiments were performed in six experimental series (*I, II, III, IV, V, VI*) as referred to in *Results.* Absolute

inhibition values can only be compared within each of these experimental series. The concentration of gammaglobulin in the incubation media was 1.6 mg/ml for the experimental series *I, II, III, IV, VI* and 0.4 mg/ml for series *V*. The protein concentration of (Na^+-K^+)-ATPase during incubation with the antibodies was 0.05 mg/ml.

To measure the dose response of (Na^+-K^+)-ATPase activity to gammaglobulin, the membrane-bound enzyme (0.075 mg/ml) was incubated for 120 min with varying concentrations of gammaglobulin (gammaglobulin preparation from experimental series VI) in the presence of 20 mM imidazole-HCl, pH 7.5, 2 mM EGTA, 1 mg/ml BSA and, in addition, phosphate, cations, and ATP as indicated in Figs. 3 and 4. Then samples were taken and the initial rates of (Na^+-K^+)-ATPase activity were measured.

Control Measurements

To determine the degree of antibody inhibition in the presence of different ligands, the (Na^+-K^+)-ATPase activities of the antibody-inhibited enzyme had to be compared to the (Na^+-K^+)-ATPase activity of the untreated enzyme in the presence of the respective ligand combinations. Therefore a control experiment without addition of gammaglobulin was performed in parallel to each inhibition experiment and the (Na^+-K^+)-ATPase activity of the antibody-inhibited enzyme was expressed as percent of the control activity. The control activities were not significantly influenced if the enzyme was preincubated in the presence of 3 or 10 mM $MgCl_2$, 3 or 10 mM $MgCl_2$ plus 150 mM NaCl and in the presence of 3 or 10 mM $MgCl_2$ plus 150 mM KCl. For the enzyme preincubation with ATP, in the first experiments ATP from equine muscle was used which was obtained from Sigma. However, reports appeared during this study [4–6] which demonstrate that Sigma ATP may modify the (Na^+-K^+)-ATPase activity. This effect, which seems to be due to the presence of vanadate in some ATP preparations which are isolated from muscular tissue [5], was not observed with ATP obtained from Boehringer (Mannheim) [4]. Thus we used Boehringer ATP in the later studies. We furthermore measured whether the (Na^+-K^+)-ATPase activity of our enzyme was modified by Sigma ATP under the preincubation conditions which were employed in the antibody inhibition studies. In contrast to Boehringer ATP which had no significant effect on the (Na^+-K^+)-ATPase activity of the enzyme, Sigma ATP reduced the (Na^+-K^+)-ATPase activity significantly if it was present together with certain cosubstrate combinations during the preincubation of the enzyme (Table 1). To find out whether the antibodies act on the modifier which is present in Sigma ATP, in some experiments the antibody inhibition in the presence of Sigma ATP and in the presence of Boehringer ATP was compared. Although the control activities were reduced in the presence of Sigma ATP and certain cosubstrate combinations, the percent values of antibody inhibition which were related to the control activities at the identical ligand combinations were the same whether ATP from Boehringer or from Sigma was used. Thus the modifier which is present in Sigma ATP does not significantly affect the antibody inhibition of the enzyme. The source of ATP is specified in each of the reported experiments.

Materials

ATP and ADP as their tris salts were either supplied by Sigma (London) or were prepared by ion exchange chromatography from the ATP and ADP sodium salts obtained from Boehringer (Mannheim). Adenylyl (β-γ-methylene) diphosphonate was also pur-

Table 1. Effect of enzyme preincubation with ATP from equine muscle (Sigma) in combination with different cosubstrates on the (Na^+-K^+)-ATPase activity

Ligand combinations present during preincubation of the enzyme (mM)	(Na^+-K^+)-ATPase activity (percent of enzyme activity obtained after preincubation in the absence of ATP and cosubstrates)
No addition	100. ± 2.2
3 ATP	99.5 ± 2.1
10 ATP	99.6 ± 2.3
3 ATP + 150 NaCl	100.3 ± 2.4
10 ATP + 150 NaCl	100.1 ± 2.3
3 ATP + 150 KCl	100.2 ± 2.2
10 ATP + 150 KCl	98.1 ± 2.3
3 $MgCl_2$ + 3 ATP	91.4 ± 2.4
10 $MgCl_2$ + 10 ATP	62.6 ± 2.5
3 $MgCl_2$ + 3 ATP + 150 NaCl	94.5 ± 2.0
10 $MgCl_2$ + 10 ATP + 150 NaCl	84.0 ± 2.1
3 $MgCl_2$ + 3 ATP + 150 KCl	61.1 ± 1.9
10 $MgCl_2$ + 10 ATP + 150 KCl	46.1 ± 2.0

Membrane-bound enzyme (0.05 mg/ml) was incubated at 0°C in a buffer containing 20 mM imidazole-HCl, pH 7.5, 2 mM EGTA, 6 mM phosphate, 1 mg/ml BSA and Sigma ATP plus different cosubstrates as indicated in the table. After 3 hr the (Na^+-K^+)-ATPase activity was measured. Mean values with SD of six measurements are given.

chased from Boehringer and the other chemicals were obtained as described previously [13].

Results

The antibody action at different combinations of ATP, Mg^{++}, Na^+, K^+ and phosphate was studied by measuring time courses of antibody inhibition and by measuring dose response curves of (Na^+-K^+)-ATPase activity to gammaglobulin. As the reactivation of the antibody-inhibited enzyme proceeds very slowly [13], the (Na^+-K^+)-ATPase activity of the enzyme, which was inhibited in the presence of various ATP and cosubstrate combinations, could be measured under standard conditions. The rate constant and the equilibrium value of enzyme inhibition are dependent on the antibody population and concentration and are furthermore dependent on the enzyme concentration and activity. The antibody action in the presence of different combinations of ATP and cosubstrates can therefore only be compared if these parameters are kept constant. This was the case in each of the performed experimental series.

The antibody inhibition at equilibrium and also the velocities of onset of the antibody inhibition were markedly affected if Mg^{++}, ATP, Na^+ and K^+ in certain combinations were added to the incubation medium. As demonstrated in Fig. 1

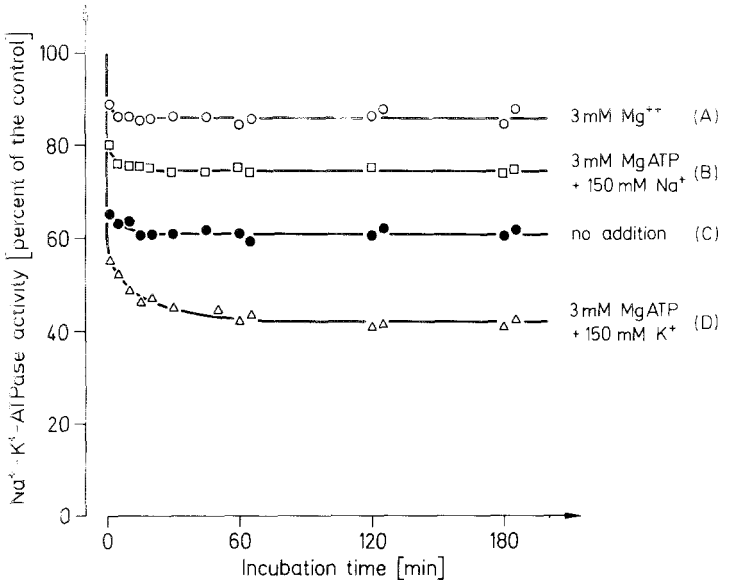

Fig. 1. Time courses of antibody inhibition of membrane-bound (Na^+-K^+)-ATPase in the presence of different ATP and cosubstrate combinations. The antigen antibody reaction was performed in a buffer containing 20 mM imidazole-HCl, pH 7.5, 6 mM phosphate, 2 mM EGTA, 1 mg/ml BSA and, in addition, ATP and cosubstrates as indicated in the figure. Dependent on the combinations of Mg^{++}, ATP, Na^+ and K^+ which were present during the antigen antibody reaction, four different degrees of antibody inhibition (*A, B, C, D*) were obtained at equilibrium. The data are presented as percent of activity of enzyme incubated under respective buffer conditions without antibodies. The ATP used in this experiment was obtained from Sigma. Experimental series *I*

and listed in Table 2, four different degrees of antibody inhibition (*A, B, C, D*) could be distinguished according to variations of the Mg^{++}, Na^+, K^+ and ATP combinations. The antibody inhibition at equilibrium followed the sequence $A < B < C < D$. From Table 2 it can be seen that this sequence was maintained in all experimental series, although the absolute values of antibody inhibition varied considerably in the different series. At the described ion and ATP combinations not only different degrees of antibody inhibition at equilibrium (*A, B, C, D*) but, as expected, also different velocities of onset of the antibody inhibition were found. The time courses of enzyme inhibition are biphasic in semilogarithmic plots (Fig. 2). When the slow phase of antibody inhibition was analyzed, it was found that the rate of (Na^+-K^+)-ATPase activity decreased exponentially with time. When the data from experiments were taken together in which combinations of ions and ATP were used which lead to the same of the four status of enzyme inhibition at equilibrium (*A* or *B* or *C* or *D*), four corresponding rates of enzyme inhibition could be distinguished (Fig. 2). For the slow phases of antibody inhibition the time required to produce half final inhibition ($t_{0.5}$) was 2.3, 5.4, 8.8, and 28.8 min for *A, B, C,* and *D,* respectively.

Effects of Phosphate, Mg^{++} and ATP on the Antibody Action

By calorimetric measurements [14] and by antibody inhibition studies [17], results have been obtained which suggest that the structural state of the (Na^+-K^+)-ATPase molecule is changed if phosphate binds to enzyme. It was therefore tested whether these structural states of the enzyme may also be detected in our system. In the absence of ATP, Mg^{++}, Na^+ and K^+ it was found that addition of 6 mM phosphate to the incubation buffer did not lead to a significant change in the degree of antibody inhibition (Fig. 4). Also if ATP, Mg^{++} and Na^+ or if Mg^{++}, ATP and K^+ were present, the addition of phosphate did not significantly influence the degree of antibody inhibition which was obtained at the equilibrium of the antigen antibody reaction (Fig. 3).

Concerning the influence of Mg^{++} and ATP on the antibody inhibition of membrane-bound enzyme it was found that the (Na^+-K^+)-ATPase activity was inhibited by the antibodies to the same extent (*C*) whether ATP, or Mg^{++} and ATP in equimolar amounts or whether none of either was present during the incubation (Table 2, exptl series *I* and *III*). If, however, Mg^{++} in the absence of ATP was present in the incubation medium the antibody inhibition of (Na^+-K^+)-ATPase activity was considerably smaller (*A*) than in the absence of Mg^{++}. This effect was observed in the absence and presence of phosphate (Table 2, exptl series *I, II, VI*; Fig. 4).

Effects of Na^+ and K^+ on the Antibody Action

The effects of Na^+ and K^+ on the antibody inhibition of the (Na^+-K^+)-ATPase activity were studied in the absence of Mg^{++} and ATP, in the presence of only ATP or only Mg^{++}, and in the presence of both Mg^{++} and ATP. Na^+ and K^+ had no effect on the degree of antibody inhibition of (Na^+-K^+)-ATPase activity (*C*) of the membrane-bound enzyme if Mg^{++} and ATP were absent (Table 2, exptl series *I, V*). Also if ATP was present at a concentration of 3 mM or 10 mM but Mg^{++} was absent, the degree of antibody inhibition (*C*) was not altered if Na^+ or K^+ were added (Table 2, exptl series *I, III*). As described above, the equilibrium value of antibody inhibition was decreased if Mg^{++} was present during the antibody action (*A*). If Na^+ or K^+ were present in addition, the same degree of antibody inhibition was obtained (Table 2, exptl series *I, V*). The respective experiments were performed at a Mg^{++} concentration of 3 mM or 10 mM. In contrast, Na^+ and K^+ strongly influence the antibody inhibition of (Na^+-K^+)-ATPase activity if both Mg^{++} and ATP are present in the incubation medium (Table 2, exptl series *I-VI*; Fig. 3). The equilibrium value of the antibody inhibition in the presence of $Mg^{++}$and ATP (*C*) is considerably increased if K^+ (*D*) and decreased if Na^+ (*B*) is also present. From Table 2 (exptl series *I, II, V, VI*) it can be seen that the antibody inhibition in the presence of Mg^{++}, ATP and Na^+ was greater (*B*) than in the presence of only Mg^{++} (*A*). The effects of Na^+ and K^+ on the antibody inhibition in the presence of Mg^{++} and ATP were equally observed whether ATP from equine muscle obtained from Sigma (Table 2) or whether ATP obtained from Boehringer (Fig. 3) was used.

Table 2. Antibody inhibition of membrane-bound (Na^+-K^+)-ATPase in the presence of different ATP and cosubstrate combinations.

Ion and ATP combinations present during the antigen antibody reaction (mM)	Percent antibody inhibition at equilibrium						Degree of inhibition
	Experimental series						
	I	II	III	IV	V	VI	
1) 3 Mg^{++}	13.9 ± 2.1 (12)	43.7 ± 1.3 (7)				40.5 ± 1.3 (7)	
2) 3 Mg^{++} + 150 Na^+	13.5 ± 1.2 (6)						
10 Mg^{++} + 150 Na^+					10.6 ± 1.1 (12)		A
3) 3 Mg^{++} + 150 K^+	13.2 ± 1.4 (5)						
10 Mg^{++} + 150 K^+					10.9 ± 1.2 (12)		
3 Mg^{++} + 3 ATP + 150 Na^+	25.3 ± 1.1 (8)			42.1 ± 1.1 (6)	16.4 ± 1.0 (6)		
10 Mg^{++} + 10 ATP + 150 Na^+		51.6 ± 2.3 (5)	18.0 ± 3.3 (8)		16.2 ± 1.5 (6)	45.1 ± 2.2 (5)	B
1) 150 Na^+	38.4 ± 0.7 (11)				21.2 ± 1.1 (7)		
2) 150 K^+	38.5 ± 1.3 (10)				20.9 ± 1.7 (8)		
3) 10 ATP	38.9 ± 0.9 (7)		32.0 ± 2.4 (11)				

4)	3 ATP + 150 Na^+	38.4 ± 1.4 (10)									C
	10 ATP + 150 Na^+	38.4 ± 1.7 (8)		30.9 ± 2.5 (8)							
5)	3 ATP + 150 K^+	38.4 ± 3.2 (7)									
	10 ATP + 150 K^+	38.7 ± 1.7 (7)		32.5 ± 1.1 (7)							
6)	10 Mg^{++} + 10 ATP	39.0 ± 1.4 (8)	59.4 ± 1.9 (6)	30.1 ± 2.2 (8)				55.5 ± 1.9 (8)			
7)	No addition	38.9 ± 1.3 (10)		31.2 ± 2.3 (6)							
	3 Mg^{++} + 3 ATP + 150 K^+	57.7 ± 1.3 (6)	71.7 ± 1.9 (6)			67.5 ± 2.7 (6)	39.8 ± 1.2 (6)				D
	10 Mg^{++} + 10 ATP + 150 K^+		72.3 ± 1.7 (6)	56.2 ± 2.6 (6)			39.2 ± 2.5 (7)	64.4 ± 1.0 (5)			

The degrees of antibody inhibition at equilibrium of the antigen antibody reaction which were obtained in a buffer containing 20 mM imidazole-HCl, pH 7.5, 2 mM EGTA, 6 mM phosphate, 1 mg/ml BSA and in addition the indicated ATP and cosubstrate combinations are compared. Data are presented as percent of activity of enzyme incubated in the respective buffer. In the table mean values with SD (number of measurements in parenthesis) are given. Data from six experimental series (*I-VI*) are presented. As identical enzyme and antibody concentrations and preparations were used within each experimental series, the influence of ATP and cosubstrates can be seen within each series. Dependent on ATP and cosubstrates present during the antigen antibody reaction, four different degrees of antibody inhibition at equilibrium (*A, B, C, D*) could be distinguished. The inhibition degree *A* was found in the presence of three (*1-3*), the degree *C* in the presence of seven (*1-7*), the degree *B* and *D* in the presence of one ATP and cosubstrate combination. In the experiments described in the table, ATP from equine muscle obtained from Sigma was used.

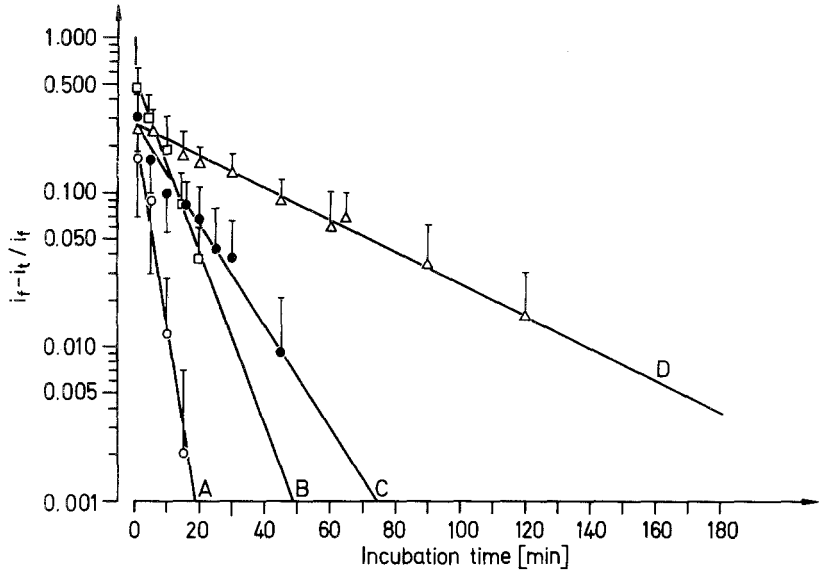

Fig. 2. Time courses of antibody inhibition of (Na^+-K^+)-ATPase activity in the presence of different ATP and cosubstrate combinations demonstrated in a semilogarithmic plot. The combinations of ions and ATP which lead to the same of the four status of enzyme inhibition at equilibrium (*A, B, C,* or *D*) were taken together and the mean values and standard deviations of the respective time courses (*A, B, C, D*) are presented. The time courses of antibody inhibition are biphasic. For the slow phases of antibody inhibition straight lines of different slopes were obtained in the semilogarithmic plots. The antibodies have been added to the membrane-bound enzyme at the time 0. At equilibrium the relative inhibition of the (Na^+-K^+)-ATPase activity i_f is reached; i_t represents the relative inhibition at the indicated time intervals. The experiments were performed with Sigma ATP

Specificity of the Na^+ and K^+ Effect on the Antibody Inhibition

The influence of Na^+ and K^+ in the presence of both Mg^{++} and ATP on the antibody inhibition of (Na^+-K^+)-ATPase activity at equilibrium was studied in more detail. It was found that, in the presence of Mg^{++} plus ATP, concentrations of K^+ higher than 10 mM were necessary to obtain the maximal K^+-induced increase of the antibody inhibition which was obtained at the equilibrium of the antigen antibody reaction (Table 3). If the K^+ concentration was increased beyond 150 mM the K^+ effect on the antibody action became smaller again. K^+ also increased the antibody inhibition of (Na^+-K^+)-ATPase activity if, in addition to Mg^{++} and ATP, Na^+ was present. It is remarkable that under these conditions about one hundred times lower K^+ concentrations were needed than those which were necessary to obtain the maximal K^+ effect in the absence of Na^+ (Table 3).

To evaluate whether the Na^+ and K^+ effect on the antibody inhibition in the presence of Mg^{++} and ATP was a ATP-specific phenomenon, ATP was replaced by the ATP analog adenylyl (β-γ-methylene) diphosphonate or by ADP. From Table 4 it can be seen that neither in the presence of Mg^{++} and adenylyl (β-γ-

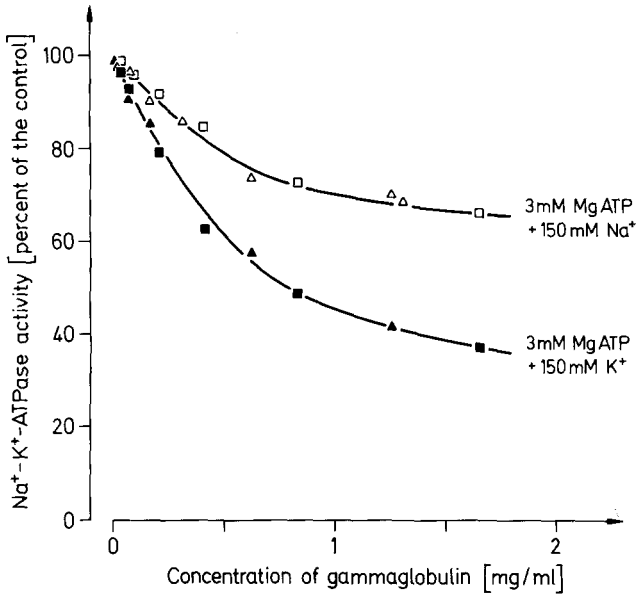

Fig. 3. Dose response of membrane-bound (Na^+-K^+)-ATPase to gammaglobulin in the presence of different ligands. Enzyme (0.075 mg/ml) was incubated with increasing concentrations of gammaglobulin in an incubation buffer containing 20 mM imidazole-HCl, pH 7.5, 2 mM EGTA, 1 mg/ml BSA and ATP and cosubstrates, as indicated in the figure (triangles). In two experiments (squares) 6 mM phosphate was additionally present in the incubation buffer. After 2 hr incubation at 0°C the (Na^+-K^+)-ATPase activity was measured. Data are presented as percent of activity of enzyme incubated in the absence of gammaglobulin under respective buffer conditions. The ATP used in this experiment was obtained from Boehringer (Mannheim). Experimental series *VI*

methylene) diphosphonate nor in the presence of Mg^{++} and ADP was an effect of Na^+ or K^+ on the antibody inhibition of the (Na^+-K^+)-ATPase activity observed. Thus, the effect of Na^+ and K^+ on the antibody inhibition of the (Na^+-K^+)-ATPase activity in the presence of Mg^{++} and ATP is indeed a highly ATP-specific phenomenon.

The Na^+ and K^+ effect on the antibody inhibition of (Na^+-K^+)-ATPase activity in the presence of Mg^{++} and ATP was measured in enzyme preparations which had previously been freed from ATP and to which afterwards ATP, Mg^{++} and Na^+ or K^+ were added simultaneously. In experiments in which the ATP which is present during the enzyme preparation was not removed, no effect of K^+ in the presence of Mg^{++} and ATP on the antibody inhibition could be detected. A possible explanation for this finding was that it might be necessary that the enzyme first binds K^+ before its configuration could be changed by Mg^{++} and ATP, which results in an altered response to the antibodies. Consequently, it was tested whether the observed antibody inhibition of the (Na^+-K^+)-ATPase activity is different if, in the presence of Mg^{++}, first K^+ or Na^+ and afterwards ATP was added to the ATP-free enzyme, or vice versa. If in the presence of Mg^{++} first K^+ and

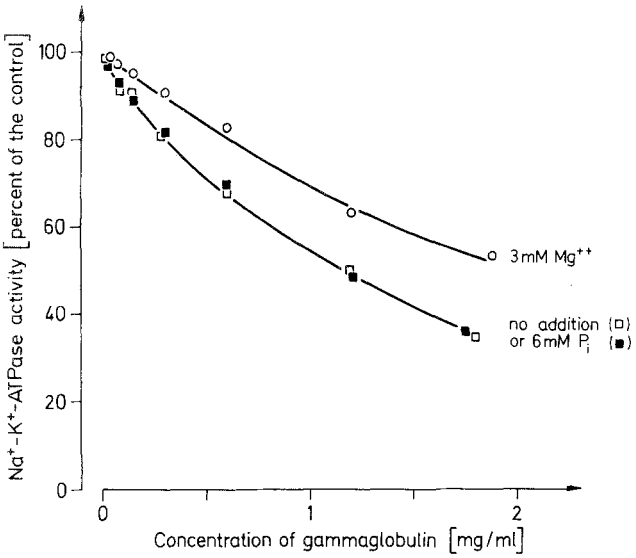

Fig. 4. Effect of Mg^{++} and phosphate on the dose response of membrane-bound (Na^+-K^+)-ATPase to gammaglobulin. The antigen antibody reaction was performed in a buffer containing 20 mM imidazole-HCl, pH 7.5, 2 mM EGTA, 1 mg/ml BSA and, in addition, 6 mM phosphate or 3 mM Mg^{++}, as indicated in the figure. The experimental conditions were analog to those of Fig. 3

Table 3. Concentration dependence of the K^+ effect on the antibody inhibition of the membrane-bound (Na^+-K^+)-ATPase in the presence of Mg^{++} plus ATP or in the presence of Mg^{++} plus ATP plus Na^+

Ion and ATP combinations			
Na^+ (mM)	MgATP (mM)	K^+ (mM)	Percent antibody inhibition at equilibrium
0	10	0	30.1 ± 2.2 (8)
0	10	1	30.2 ± 3.1 (7)
0	10	10	39.0 ± 4.4 (6)
0	10	150	56.2 ± 2.6 (6)
0	10	500	29.4 ± 2.9 (8)
150	10	0	18.0 ± 3.3 (8)
150	10	0.02	31.7 ± 1.9 (8)
150	10	0.1	41.4 ± 2.2 (6)
150	10	150	39.7 ± 1.2 (9)

The antibody inhibition was measured at equilibrium of the antigen antibody reaction. The data which were obtained from experimental series *III* are presented as in Table 2.

Table 4. Nucleotide specificity for the effects of Na^+ and K^+ on the antibody inhibition of the membrane-bound (Na^+-K^+)-ATPase measured at the equilibrium of the antigen antibody reaction

Nucleotide and cosubstrate combinations				
	Cosubstrates			
Nucleotides (mM)	Mg^{++} (mM)	Na^+ (mM)	K^+ (mM)	Percent antibody inhibition at equlibrium
3 ATP	3	150	0	16.4 ± 1.0 (6)
10 ATP	10	150	0	16.6 ± 2.7 (7)
3 ATP	3	0	150	39.8 ± 1.2 (6)
10 ATP	10	0	150	39.2 ± 2.5 (7)
3 ADP	3	150	0	17.6 ± 1.7 (7)
10 ADP	10	150	0	16.6 ± 2.7 (7)
3 ADP	3	0	150	16.4 ± 1.5 (7)
10 ADP	10	0	150	17.3 ± 3.5 (9)
3 AMP-PCP	3	150	0	16.7 ± 2.4 (9)
3 AMP-PCP	3	0	150	16.5 ± 2.1 (8)

The experiments are presented as in Table 2. Experimental series *V*.

then ATP was added to the enzyme, the antibody inhibition which was started 15 min later was as strong as if K^+ and ATP had been added simultaneously. If, however, ATP and Mg^{++} were added prior to K^+, the antibody inhibition was considerably less, showing no difference from the antibody inhibition in the presence of Na^+, Mg^{++} and ATP (Fig. 5). In the case of Na^+ the antibody inhibition of (Na^+-K^+)-ATPase was not significantly different if, in the presence of Mg^{++}, Na^+ was added prior to ATP, vice versa, or if Na^+, Mg^{++}, and ATP were added together (Fig. 5*a*). Thus it appears that the antibody response of the membrane-bound (Na^+-K^+)-ATPase, in the presence of Mg^{++}, ATP, and K^+ but not in the presence of Mg^{++}, ATP, and Na^+, is dependent on the sequence in which ATP and the cation has been bound to the enzyme.

Furthermore, it was tested whether the antibody inhibition of the (Na^+-K^+)-ATPase activity of the solubilized enzyme could be affected in the same way as that of the membrane-bound enzyme. From Table 5 it can be seen that the antibody inhibition of the (Na^+-K^+)-ATPase activity of the solubilized enzyme was identical whether—in the absence of Na^+, K^+, and ATP—Mg^{++} was present or not. Also the effects of Na^+ and K^+ on the antibody inhibition in the presence of Mg^{++} and ATP could not be detected with the solubilized (Na^+-K^+)-ATPase.

Discussion

Interpretations about the Mode of Antibody Inhibition

To interpret our results properly the mechanism of inhibition of the (Na^+-K^+)-ATPase activity by our antibodies must be considered. In previous experiments

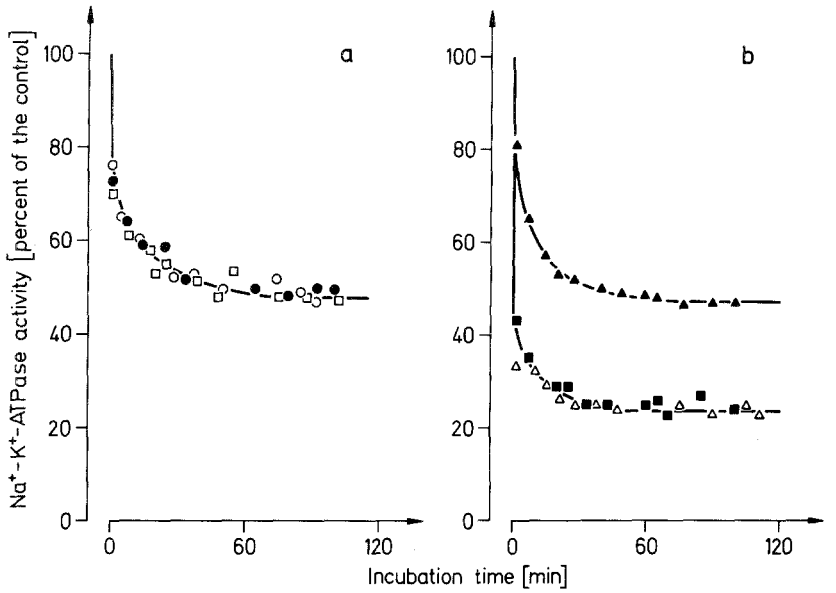

Fig. 5. Effect of the sequence of addition of ATP and Na^+ or K^+ to the membrane-bound enzyme on the antibody inhibition. (*a*): In the presence of Mg^{++} first Na^+, then 15 min later ATP, and after another 15 min the antibodies were added to the enzyme (o-o); in the presence of Mg^{++} first ATP, 15 min later Na^+, and after another 15 min the antibodies were added (□-□); in the presence of Mg^{++}, ATP together with Na^+ and 30 min later the antibodies were added to the enzyme (●-●). (*b*): In the presence of Mg^{++} first K^+, then 15 min later ATP, and after another 15 min the antibodies were added (△—△); in the presence of Mg^{++} first ATP, 15 min later K^+, and after another 15 min the antibodies were added (▲—▲); in the presence of Mg^{++}, K^+ and ATP together and 30 min later the antibodies were added to the enzyme (■—■). During antigen antibody reaction the incubation buffer contained 20 mM imidazole-HCl, pH 7.5, 6mM phosphate, 2 mM EGTA, 10 mM $MgCl_2$, 10 mM ATP, 1 mg/ml BSA and 150 mM NaCl (*a*) or 150 mM KCl (*b*). For these experiments Sigma ATP was used. Experimental series *VI*

[13] we obtained similar kinetics for the antibody effect on the ATP dependence of the (Na^+-K^+)-ATPase activity and for the antibody effect on the ATP binding to the enzyme. Therefore, it seemed probable that the antibodies inhibit the overall (Na^+-K^+)-ATPase reaction at the ATP binding step.

The antibodies may inhibit the ATP binding by direct interaction or by means of a conformational change at the ATP binding site(s). Such a conformational change may be induced by antibody binding to the enzyme protein or also possibly by antibody binding to lipids which are closely associated to (Na^+-K^+)-ATPase protein and which are not removed by the solubilization with Lubrol. As the ATP binding sites are located at the inner surface of the cell membrane, it seems probable that the antibodies inhibit the ATP binding and the (Na^+-K^+)-ATPase activity from this membrane side. Experiments from Jørgensen and coworkers [12], which demonstrate that (Na^+-K^+)-ATPase antibodies inhibit the ouabain-sensitive outflux of Na^+ from red cell ghosts only if the antibodies were ap-

Table 5. Equilibrium values of antibody inhibition of solubilized (Na^+-K^+)-ATPase in the presence of some ATP and cosubstrate combinations

Ion and ATP combinations (mM)	Percent antibody inhibition at equilibrium
3 Mg^{++}	28.5 ± 1.4 (8)
No addition	29.0 ± 1.7 (9)
10 Mg^{++} + 10 ATP + 150 Na^+	29.5 ± 1.7 (8)
	28.9 ± 2.5 (7)
10 Mg^{++} + 10 ATP + 150 K^+	28.5 ± 1.2 (7)
	30.5 ± 2.5 (6)

The experiments were performed as described in Table 2.

plied from the inner side of the membrane, support this view. Because of the size of gammaglobulin molecules, it can be assumed that the antibodies bind to the surface of the (Na^+-K^+)-ATPase protein or to the surface of closely associated lipids.

It has been shown above that the antibody inhibition of the (Na^+-K^+)-ATPase activity of the membrane-bound enzyme is influenced by the presence of different combinations of ATP, Mg^{++}, Na^+, and K^+. At the equilibrium of the antigen antibody reaction, four different states of enzyme inhibition could be distinguished. Further, it was found that the velocity by which the antibody inhibition started was different at ATP and ion combinations which led to different degrees of equilibrium inhibition.

In principle, the different characteristics of antibody action at different ATP and ion combinations could be explained by changes of the enzyme or of the antibody structure. However, an effect of the ATP and cosubstrate combinations on the antibodies can be excluded as reason for the different inhibition degrees of the (Na^+-K^+)-ATPase activity: The different degrees of antibody inhibition were not observed if the (Na^+-K^+)-ATPase was altered by taking the enzyme out of the membrane by solubilization or if the enzyme was altered by changing the sequence in which K^+ and ATP were added during preincubation. Further, it is the enzyme substrate, ATP, and the cosubstrates of the (Na^+-K^+)-ATPase which must be present in distinct combinations to exhibit and influence on the antibody inhibition of the (Na^+-K^+)-ATPase activity. Thus our results suggest that under the described conditions the membrane-bound (Na^+-K^+)-ATPase exhibits different states of the enzyme structure. It cannot, however, be excluded that closely associated lipids may be included in these structural changes of the enzyme.

Our finding that the equilibrium values of antibody inhibition were reached more slowly if higher degrees of antibody inhibition were obtained at equilibrium can only be tentatively explained, as the biphasic character of the inactivation curves is not fully understood [13] and as we have no data concerning the effect of different ligand combinations on the reactivation of the antibody-inhibited enzyme. A possible explanation for the obtained inactivation curves is that structural changes of the (Na^+-K^+)-ATPase molecule may mainly affect the reactivation of the antibody-inhibited enzyme. Thus the rate constant of enzyme reactivation may be reduced drastically, while the rate constant of enzyme in-

activation may be reduced to a smaller extent. Therefore, the antibody inhibition at equilibrium may be increased although the rate constant of enzyme inhibition is reduced.

Effect of Mg^{++} and Phosphate on the (Na^+-K^+)-ATPase Structure

It has been demonstrated above that the presence of Mg^{++} ions can influence the characteristics of antibody inhibition of the membrane-bound (Na^+-K^+)-ATPase. In the presence of Mg^{++} the antibody inhibition at equilibrium was diminished and the rate of the slow phase of the enzyme inactivation was increased. This Mg^{++} effect was also observed if phosphate, Na^+, or K^+ were present in addition but was no longer seen if ATP was present in the incubation buffer.

Antibody studies [17,18] and several studies in which different methods were employed [1-3, 8, 14, 21, 24] have been reported which suggest, in parallel to our findings, that Mg^{++} induces a conformational change of the enzyme. In addition, data were reported from which it may be concluded that the binding of phosphate also leads to a structural change of the (Na^+-K^+)-ATPase [14, 17, 18]. Thus, McCans and coworkers [17], who measured the influence of Mg^{++} and of phosphate on the inhibition of the (Na^+-K^+)-ATPase activity by different fractions of their (Na^+-K^+)-ATPase antibodies, found that the enzyme inhibition by one of the tested antibody fractions was decreased if Mg^{++} was present and — at variance with our results —was also decreased if phosphate was present. The Mg^{++} effect on the antibody inhibition, however, was not detected with their antibodies if phosphate was present in the incubation buffer. Measuring the rate and the maximal degree of (H^3) ouabain binding to the enzyme, it was recently demonstrated [18] that in the presence of Mg^{++} phosphate influenced the antigen antibody reaction between (Na^+-K^+)-ATPase and specific antibodies.

Furthermore, it has been shown that the destruction of (Na^+-K^+)-ATPase activity by trypsin was smaller if Mg^{++} was added [24]. In addition, it has been demonstrated that the ouabin-induced inhibition of the phosphorylation [21] and of the ATP hydrolyzing activity [1] is increased in the presence of Mg^{++}. Moreover, it was found that the inhibitory effect of N-ethylmaleimide (NEM) on the phosphorylation of the (Na^+-K^+)-ATPase was increased but that the inhibitory effect of NEM on the K^+-dependent dephosphorylation and on the ADP-ATP exchange was decreased if Mg^{++} was present during the incubation with NEM [2, 3]. Along the same line are the findings of Kuriki and coworkers [14] who performed calorimetric measurements which demonstrate enthalpy changes associated with the binding of Mg^{++} or phosphate. Furthermore, it has been recently demonstrated that the fluorescence of dimethylaminonaphthalene sulfonyl labeled (Na^+-K^+)-ATPase was increased if Mg^{++} was added [8].

The different methods by which the Mg^{++} or phosphate-induced changes in the enzyme conformation have been sensed give different information. Thus, by calorimetric measurements, only conformational changes which are accompanied by enthalpy changes can be found. With SH reagents, probably structural changes in the vicinity of the ATP binding site(s) are detected because there is

some evidence that a sulfhydryl group may exist in the ATP binding site(s) [19]. By ouabain binding or ouabain inhibition studies, only structural changes in the vicinity of the ouabain binding site(s) can be sensed. As the amino acid sequence and the conformation of the (Na^+-K^+)-ATPase is not yet known, structural changes which are detected by trypsin digestion experiments can, at the moment, hardly be characterized. In contrast to the other methods, by antibody studies only structural changes are detected which concern the surface of the (Na^+-K^+)-ATPase. Moreover, if the antibody inhibition studies are performed at a membrane-bound enzyme as in our experiments only those changes can be sensed which concern parts of the (Na^+-K^+)-ATPase molecule which are not hidden in the membrane.

The Mg^{++}-induced conformational change of the (Na^+-K^+)-ATPase seems to be drastic. It is accompanied by a large change of enthalpy [14]. The structural change includes protein regions in the vicinity of the ATP binding site(s) as well as in the vicinity of the ouabain binding site(s) [1-3, 21]. From our results it may be further assumed that a region of the (Na^+-K^+)-ATPase molecule is involved which is accessible from the inner membrane surface. If phosphate binds to the (Na^+-K^+)-ATPase, the enzyme structure is probably altered in a different manner. This conformational change is also accompanied by a large change of enthalpy [14], but it can be distinguished from the enzyme structure in the presence of Mg^{++} by antibody inhibition studies [17, 18]. The finding that a phosphate effect on the antibody inhibition was only observed with one of the antibody fractions tested by McCans and coworkers [17] and was not found with our antibodies demonstrates that antibodies raised against (Na^+-K^+)-ATPase consist of different antibody populations which may bind to different regions of the enzyme and may inhibit the (Na^+-K^+)-ATPase activity in different ways.

Effect of Na^+ and K^+ on the (Na^+-K^+)-ATPase Structure

Previous studies with different inhibitors of the (Na^+-K^+)-ATPase suggest that Na^+ and K^+ can induce different structural states of the (Na^+-K^+)-ATPase if ATP has also bound to the enzyme. Thus, in different laboratories [2, 3, 9, 22] it has been found that the (Na^+-K^+)-ATPase is inactivated by NEM in the presence of ATP and K^+ but protected if ATP and Na^+ are present during the incubation with NEM. Furthermore, it has been shown that ouabain binding was increased by addition of Na^+ and decreased by addition of K^+ if Mg^{++} and ATP [16] or if only ATP [1] was present during ouabain incubation. In addition, it has been demonstrated that the inhibitory effect of (Na^+-K^+)-ATPase antibodies on the binding of ouabain to the enzyme was modified if Na^+ was added in the presence of Mg^{++} and ATP [18].

In contrast, trypsin digestion experiments have been reported by Somogyi [24] and Jørgensen [11], which suggest that also in the absence of ATP the conformation of the (Na^+-K^+)-ATPase may be altered by Na^+ and K^+. In these experiments it was found that Na^+ and K^+ influence the time course of trypsin inactivation of the (Na^+-K^+)-ATPase activity in the absence of ATP [11, 24] and also the degradation pattern of the high molecular weight subunit of the enzyme

[11]. Trying to repeat the trypsin inactivation experiments which were reported by Jørgensen with our enzyme[2], we obtained similar results—demonstrating an effect of Na^+ and K^+ in the absence of ATP—only if the nucleotides had been removed from the enzyme by centrifugation in the absence of $MgCl_2$ as it was done in Jørgensen's experiments. If, however, the nucleotides were removed by gel filtration in the presence of glycerol and $MgCl_2$—a method which we applied routinely—Na^+ and K^+ did not influence the trypsin inactivation of the (Na^+-K^+)-ATPase activity[3]. In modified trypsin digestion experiments[4] in which the glycerol-treated enzyme was employed, we further found that the degradation pattern of the low molecular weight subunit was only altered by the addition of Na^+ and K^+ if ATP or if Mg^{++} plus ATP were present in addition. Thus we would support the view that ATP is necessary to modulate the enzyme structure in the presence of Na^+ or K^+. Furthermore, our antibody inhibition experiments suggest that, in the presence of Na^+ or K^+, MgATP has a different effect on the (Na^+-K^+)-ATPase structure than ATP alone. Our finding that the high degree of antibody inhibition in the presence of Mg^{++}, ATP, and K^+ was only found if K^+ and ATP were added to the enzyme in the right order—namely, first K^+ and then ATP—suggests that it is actually the structure of the K^+-enzyme complex which is altered if MgATP binds, in addition.

For the influence of Na^+ and K^+ on the antibody inhibition of (Na^+-K^+)-ATPase activity, a high degree of specificity could be demonstrated. The K^+ effect on the antibody inhibition can be seen in the presence and absence of Na^+, but the K^+ concentration which is necessary to observe a maximal effect on the antibody inhibition is about a hundredfold lower if Na^+ is present, too. Na^+ and K^+ have no effect on the antibody inhibition of the (Na^+-K^+)-ATPase if Mg^{++} and ADP or Mg^{++} and adenylyl (β-γ-methylene) diphosphonate are present. Moreover, the K^+-effect on the antibody inhibition can only be observed if in the presence of Mg^{++} first K^+ and then ATP are added to the enzyme. Thus it can be assumed that a Na^+-and K^+-specific change of the enzyme structure is recorded which occurs only if also Mg^{++} and ATP bind to the enzyme. In this conformational change a surface region which is accessible from the inner membrane seems to be included. From our trypsin digestion experiments[5] it can be concluded that both subunits of the enzyme take part in this conformational change, as either their tertiary structure or their spatial relationship to the membrane and/or to each other is altered.

Above we reported that the solubilized enzyme which also obtains a high specific Na^+-, K^+- and Mg^{++}-dependent ATPase activity does not show the Na^+-and K^+-induced differences in the antibody inhibition which can be demonstrated in the membrane-bound enzyme. Thus the Na^+-K^+-ATPase structure as sensed by the antibody inhibition experiments is only altered by Na^+ or K^+— in combination with Mg^{++} plus ATP—if the enzyme molecule is embedded in the membrane. The solubilized enzyme which has retained its hydrolytic activity may

[2]Koepsell, H. 1978. Conformational changes of membrane-bound (Na^+-K^+)-ATPase as revealed by trypsin digestion (unpublished).

[3]*Ibid.*

[4]*Ibid.*

[5]*Ibid.*

have lost the ability to obtain these Na^+- and K^+-induced structural states. The Na^+-and K^+-induced MgATP-dependent alterations of the membrane-bound enzyme may possibly represent functional states of the (Na^+-K^+)-ATPase which may be related to the transport of Na^+ and K^+.

I express my gratitude to Professor Dr. K. J. Ullrich for his continuous help and encouragement during the study. The skilful and conscientious technical assistance of R. Göpfert is gratefully acknowledged.

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