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THE EFFECT OF PROTEASES ON THE ($Na^+ + K^+$)-ACTIVATED ADENOSINE TRIPHOSPHATASE SYSTEM OF RAT BRAIN

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SUMMARY

A 10-min incubation of the ($Na^+ + K^+$)-activated ATPase preparation with 20 μg trypsin per mg protein at 25° decreased ATPase activity by approx. 50%, whereas only 10–15-fold greater concentrations of subtilisin A and of chymotrypsin decreased the activity to the same extent. Yet the simultaneously measured proteolysis was 5–6-fold more with subtilisin A or with chymotrypsin than with trypsin.

The loss in ATPase activity with proteases was partly counteracted by Mg^{2+}, Na^+, and K^+. The protective effect of cations against trypsin could be modified by ATP: the action of Mg^{2+} was completely abolished; the protection by K^+, in the presence of Mg^{2+}, was considerably lessened by ATP, whereas, under similar conditions, the effect of Na^+ was not influenced at all by ATP.

The experiments suggest that trypsin, in contrast to chymotrypsin and subtilisin A, acts at the region of the active centre of ATPase. The effect of cations and ATP are manifested by a change in the conformation of the ATPase molecule; the conformation is different in the presence of Mg^{2+} + ATP + Na^+ and in the presence of Mg^{2+} + ATP + K^+. These changes in conformation of the enzyme system seem to constitute the basis of the active ion transport across the cell membrane. It is likely that arginine or lysine has an important functional role in the active centre of ($Na^+ + K^+$)-activated ATPase.

INTRODUCTION

In spite of the many investigations of the membrane-bound ($Na^+ + K^+$)-activated ATPase, the exact mechanism of Na^+ and K^+ transport, catalyzed by this enzyme system, still remains to be clarified. Recently, the role of possible structural changes in the enzyme system during the translocation of Na^+ and K^+ has been considered[1–4].

In the present study the decrease of ($Na^+ + K^+$)-activated ATPase activity caused by different proteases and the influence of Mg^{2+}, Na^+, K^+ and adenine nucleotides on this effect have been investigated. The results suggest that ATP and monovalent cations change the conformation of the enzyme system, and that trypsin, in contrast

to other proteases, splits peptide linkages at the region of the active centre of ($Na^+ + K^+$)-activated ATPase.

METHODS

The ($Na^+ + K^+$)-activated ATPase, free from the Mg^{2+}-activated ATP-hydrolyzing enzyme, was prepared from rat brain, as described previously[5,6] apart from a single washing of the final pellet (performed with 0.25 M sucrose containing 0.1 mM EDTA and 5 mM Tris–chloride buffer (pH 7.4)).

Incubation of the enzyme preparation was carried out with different proteases at 25° in a 1.0-ml vol. containing 25 mM Tris–chloride buffer (pH 7.4 and 8.0, respectively), 1.0 mg ATPase protein and the substances to be tested. After incubation the reaction with trypsin was stopped by addition of 20 μg soy-bean trypsin inhibitor. No inhibitor was used in the case of chymotrypsin and subtilisin A. In these cases the ATPase activity was assayed immediately after the incubation had been completed.

The ATPase activity of incubated samples was determined in a 2.0-ml test volume containing 50 mM Tris–chloride buffer (pH 7.4), 5 mM Mg^{2+}, 5 mM ATP (Tris salt), 100 mM Na^+ and 20 mM K^+. After 10 min incubation at 37°, 1.0 ml of 20% trichloroacetic acid was added and the amount of P liberated was estimated by the method of LOHMANN AND JENDRASSIK[7]. ATPase activity was expressed as μmole P per 10 min per 0.2 mg ATPase protein.

The digestion of the ATPase preparation in the presence of different proteases was detected either by the pH-stat procedure or by the estimation of acid-soluble split products. The pH-stat measurements were carried out at pH 7.4 for trypsin or subtilisin A and at pH 8.0 for chymotrypsin, at 25° with a Radiometer TTT 1, + SBU/SBR 2 assembly. 10 mg ATPase protein were digested in 10 ml vol. containing 20 μg/ml trypsin, or 200 μg/ml subtilisin A, or 300 μg/ml chymotrypsin. After preliminary adjustment of the pH of the protease-free assay system, the steadiness of the pH was observed for 20 min during continuous flushing of the gas phase with N_2. The solutions of proteases were also adjusted to the desired pH prior to use. Titrations were performed with 0.01 M NaOH.

For absorbance measurements of acid-soluble split products, 1.0 mg ATPase protein was incubated at 25° in a 1.0-ml vol. containing 25 mM Tris–chloride buffer and 20 μg trypsin, 200 μg subtilisin A, 300 μg chymotrypsin, respectively. The pH of the buffer was 7.4 for trypsin and subtilisin A, and 8.0 for chymotrypsin. After the completion of the incubation 1.0 ml 10% trichloroacetic acid was added to the media and the absorbance of the protein-free filtrate was determined in a 0.5-cm cell at 280 mμ.

Trypsin (2 × crystallized, salt free; Sigma, U.S.A.) was dissolved in 0.001 M HCl and neutralised prior to use. Soy-bean trypsin inhibitor (5 × crystallized) and subtilisin A (crystalline) were products of Serva (Germany), α-chymotrypsin (2 × crystallized) was purchased from Boehringer (Germany) and from Reanal (Hungary).

RESULTS

Effect of incubation with proteases on the ($Na^+ + K^+$)-activated ATPase activity

Incubation of the ATPase preparation with trypsin, chymotrypsin or subtilisin A at 25° caused a steady loss in ATPase activity (Fig. 1).

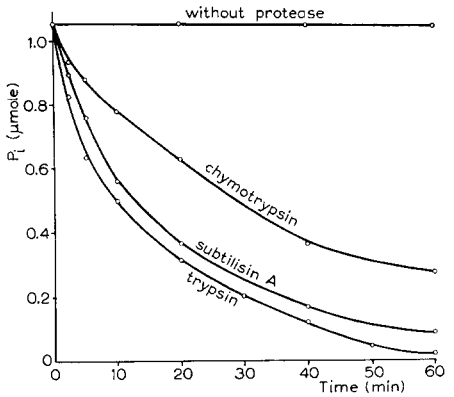

Fig. 1. Activity of the (Na+ + K+)-activated ATPase after incubation with trypsin, subtilisin A and chymotrypsin. 1.0 mg ATPase protein was incubated at 25° in a 1.0-ml sample containing 25 mM Tris–chloride buffer and 20 μg trypsin, 200 μg subtilisin A, 300 μg chymotrypsin, respectively. The pH of buffer was 7.4 for trypsin and subtilisin A, and 8.0 for chymotrypsin. After the completion of the incubation the ATPase activity of 0.2 ml incubated suspension was estimated as described in METHODS. ATPase activity: μmole P_i/10 min per 0.2 mg protein.

The decrease in ATPase activity was approx. 50% after 10 min incubation in the presence of either 20 μg trypsin or 200 μg subtilisin A. In the case of 300 μg chymotrypsin the same decrease was observed only after 30 min. Incubation of the ATPase preparation in the absence of proteases at 25° had no effect on the ATPase activity in the course of 60 min.

On the other hand, the simultaneously estimated proteolytic digestion rate of the ATPase preparation was 5–6-fold higher in the presence of chymotrypsin or subtilisin A than in the presence of trypsin as measured either by pH-stat (Fig. 2a) or as acid-soluble split products (Fig. 2b).

If the enzyme preparation was incubated in the absence of proteases and the required amount of protease was added directly to the ATPase assay system, no loss

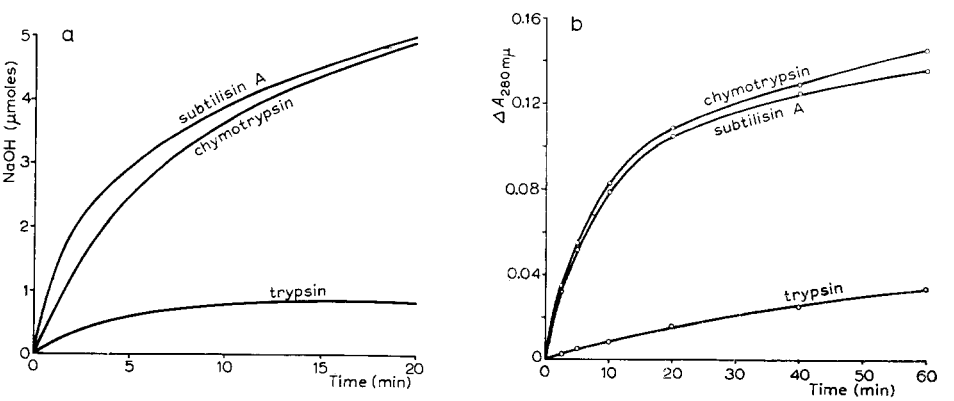

Fig. 2. a. Digestion of ATPase preparation by trypsin, subtilisin A and chymotrypsin; pH-stat records. For experimental details see METHODS. b. Absorbance of acid-soluble split products after digestion of ATPase preparation by trypsin, subtilisin A and chymotrypsin. For experimental details see METHODS.

TABLE I

ACTIVITY OF THE $(Na^+ + K^+)$-ACTIVATED ATPASE AFTER INCUBATION WITH PROTEASES

1.0 mg ATPase protein was incubated at 25° for 10 min in a 1.0-ml sample containing 25 mM Tris–chloride buffer either with (20 μg trypsin or 200 μg subtilisin A or 300 μg chymotrypsin) or without protease. The pH of the buffer was 8.0 for chymotrypsin, in the other cases it was 7.4. When incubated in the absence of proteases, the required amounts of proteases (4 μg trypsin, 40 μg subtilisin A, 60 μg chymotrypsin, respectively) were added directly to the ATPase assay system. After the completion of the incubation the ATPase activity of 0.2 ml incubated suspension was estimated. Other experimental conditions as for Fig. 1.

Conditions	*$(Na^+ + K^+)$-activated ATPase activity after incubation with protease*	
	In incubating medium	*In assay system*
No protease	1.06	1.06
Trypsin	0.52	1.01
Subtilisin A	0.56	0.96
Chymotrypsin	0.78	0.99

in the ATPase activity was found (Table I). This indicated that components of the assay system prevented the decrease of ATPase activity.

The protective effect of cations against proteases

The protection of ATPase against proteases was different in the presence of either Mg^{2+} or Na^+ or K^+ (Table II). Both Na^+ and K^+ protected against trypsin, subtilisin A and chymotrypsin. The protective effect of Mg^{2+} was observed only with trypsin. However, the protection of ATPase against this protease by K^+ was more marked than by the other two cations.

The protective effect of Mg^{2+}, Na^+ or K^+ could not be a consequence of the inhibition of trypsin itself, since these ions did not decrease its action on a synthetic substrate such as benzoylarginine-*p*-nitroanilide. Similarly, the tryptic digestion of proteins of the ATPase preparation was not decreased by Mg^{2+}, Na^+ or K^+ as measured

TABLE II

ACTIVITY OF THE $(Na^+ + K^+)$-ACTIVATED ATPASE AFTER INCUBATION WITH TRYPSIN, SUBTILISIN A AND CHYMOTRYPSIN; EFFECTS OF Mg^{2+}, Na^+ AND K^+

Incubation of ATPase preparation with different proteases was performed at 25° for 20 min either in the absence of added ions or in the presence of 2.5 mM Mg^{2+}, 100 mM Na^+, 100 mM K^+, respectively. Other experimental conditions as in the legend of Fig. 1.

Conditions	*$(Na^+ + K^+)$-activated ATPase activity after incubation with protease +*			
	–	*Mg^{2+}*	*Na^+*	*K^+*
No protease	1.04	1.02	1.04	1.04
Trypsin, 20 μg	0.34	0.56	0.51	0.75
Subtilisin A, 200 μg	0.37	0.32	0.68	0.64
Chymotrypsin, 300 μg	0.62	0.31	0.83	0.82

TABLE III

ACTIVITY OF THE $(Na^+ + K^+)$-ACTIVATED ATPASE AND ABSORBANCE OF ACID-SOLUBLE SPLIT PRODUCTS AFTER INCUBATION WITH TRYPSIN; EFFECTS OF Mg^{2+}, Na^+ AND K^+

1.0 mg ATPase protein was incubated at 25° for 20 min in a 1.0-ml sample containing 25 mM Tris–chloride buffer (pH 7.4), 20 μg trypsin and the indicated ions. After incubation the ATPase activity was assayed as in Fig. 1. For absorbance measurements see METHODS.

Added ions	*$(Na^+ + K^+)$-activated ATPase activity*	*$A_{280\ m\mu}$*
No ions	0.34	0.021
Mg^{2+}, 2.5 mM	0.58	0.023
Na^+, 100 mM	0.54	0.022
K^+, 100 mM	0.79	0.021

either by the acid-soluble split products (Table III) or by the pH-stat procedure. This presumably, points to the existence of a large amount of non-ATPase protein in the enzyme preparation. Incubation of the preparation with trypsin, either alone or in the presence of K^+, did not result in any solubilisation of ATPase activity.

Effect of ATP on the protective action of Mg^{2+}, Na^+ and K^+

The loss of ATPase activity due to trypsin was not affected by ATP alone. Similarly, the protective effect of Na^+ or K^+ was not influenced by ATP alone (Table IV). However, the protective action of Mg^{2+} was completely abolished by ATP. This

TABLE IV

ACTIVITY OF THE $(Na^+ + K^+)$-ACTIVATED ATPASE AFTER SIMULTANEOUS INCUBATION WITH TRYPSIN AND ATP; EFFECTS OF Mg^{2+}, Na^+ AND K^+

Incubation of enzyme preparation was carried out at 25° for 20 min in a 1.0-ml sample containing 25 mM Tris–chloride buffer (pH 7.4), 1.0 mg ATPase protein, 20 μg trypsin and Mg^{2+}, Na^+, K^+ and ATP, as indicated. Other experimental conditions as in the legend of Fig. 1.

Added ions	*$(Na^+ + K^+)$-activated ATPase activity after incubation*	
	Without ATP	*With 2.5 mM ATP*
No trypsin, no ions	1.04	1.04
No ion	0.34	0.34
Mg^{2+}, 2.5 mM	0.56	0.35
Na^+, 100 mM	0.51	0.54
K^+, 100 mM	0.73	0.71
Mg^{2+}, 2.5 mM + Na^+, 100 mM	0.60	0.62
Mg^{2+}, 2.5 mM + K^+, 100 mM	0.77	0.51

phenomenon was also observed in the case of a 1:1 molar ratio of Mg^{2+} to ATP and in the case of a molar excess of Mg^{2+} (Table V).

In the presence of either Na^+ or K^+, addition of Mg^{2+} + ATP yielded different results. When incubated with trypsin + Mg^{2+} + ATP + Na^+, the decrease of ATPase

TABLE V

ACTIVITY OF THE $(Na^+ + K^+)$-ACTIVATED ATPASE AFTER INCUBATION WITH TRYPSIN; SIMULTANEOUS EFFECTS OF Mg^{2+} AND ATP

1.0 mg ATPase protein was incubated at 25° for 20 min in a 1.0-ml sample containing 25 mM Tris–chloride buffer (pH 7.4), 20 μg trypsin and Mg^{2+}, ATP, as indicated. Other experimental conditions as in the legend of Fig. 1.

Added		*$(Na^+ + K^+)$-activated ATPase activity after incubation*
Mg^{2+} (mM)	*ATP (mM)*	
0	0	0.33
1.25	0	0.53
2.5	0	0.58
0	1.25	0.33
0	2.5	0.34
1.25	1.25	0.34
2.5	1.25	0.34
2.5	2.5	0.33

activity due to ATP was not observed. On the other hand, K^+, unlike Na^+, did not abolish a further loss in the ATPase activity caused by ATP + Mg^{2+} (Table IV).

In the presence of Mg^{2+} + trypsin, ADP and AMP replaced ATP but their effects were smaller than that of ATP (Table VI).

TABLE VI

ACTIVITY OF THE $(Na^+ + K^+)$-ACTIVATED ATPASE AFTER SIMULTANEOUS INCUBATION WITH TRYPSIN + Mg^{2+} AND DIFFERENT ADENINE NUCLEOTIDES

Incubation of enzyme preparation was performed at 25° for 20 min in a 1.0-ml sample containing 25 mM Tris–chloride buffer (pH 7.4), 1.0 mg ATPase protein, 20 μg trypsin, 2.5 mM Mg^{2+} and 2.5 mM adenine nucleotide as indicated. Other experimental conditions as in the legend of Fig. 1.

Added nucleotide	*$(Na^+ + K^+)$-activated ATPase activity*
No trypsin, no nucleotide	0.99
No nucleotide	0.52
ATP	0.33
ADP	0.38
AMP	0.41

DISCUSSION

Incubation of the ATPase preparation with different proteases caused a progressive loss in the ATPase activity. The loss was partially counteracted by Mg^{2+}, Na^+ or K^+. This is in agreement with the observation of GREEN AND MASIAK[8], who found that Na^+ or K^+ protected the ATPase activity against trypsin in human erythrocyte stroma.

The tryptic hydrolysis both of native and denatured serum albumin and lactalbumin was inhibited by Na^+ or K^+, if their concentration were higher than 0.1 M

(see ref. 9). Similarly the tryptic digestion of myosin was remarkably dependent on the ionic environment[10]. In our experiments the overall tryptic hydrolysis of the ATPase preparation was not decreased by cations in the concentration employed. Presumably, these cations protected only those peptide bonds which were required for ATPase activity. However, protection of peptide bonds may occur only if the cations modified the steric accessibility of the ATPase molecule to trypsin.

SKOU AND HILBERG[4] suggested that ATP caused a structural change in the ATPase. In our experiments, ATP was only effective in influencing the loss of ATPase activity due to trypsin if Mg^{2+} was also present, indicating the necessity of Mg^{2+} for binding ATP to the enzyme. The binding of ATP may expose peptide bonds which were not accessible to trypsin in the presence of Mg^{2+} alone. In the presence of Mg^{2+}, ATP lowered the protection by K^+ while the effect of Na^+, on the other hand, was not influenced at all by Mg^{2+} + ATP. It follows that the conformation of ATPase must be different in the presence of Mg^{2+} + ATP + Na^+ and Mg^{2+} + ATP + K^+.

Kinetic analysis of the $(Na^+ + K^+)$-activated ATPase indicates that the enzyme system has two sites with different affinities for Na^+ and K^+ (see refs. 1, 11, 12). The fact that the enzyme system is activated by Na^+ and K^+ on different sides of the membrane[1] suggests rotation of the active centre of the enzyme protein. It is likely that conformational changes exerted by ATP and by the transported monovalent cations in the membrane-bound $(Na^+ + K^+)$-activated ATPase, constitute the basis of the rotation of the active centre of the enzyme system bringing about the vectorial translocation of Na^+ and K^+.

The tryptic digestion of myosin yields subunits without any loss in the original ATPase activity[13–16]. The active centre in the myosin is thus resistant to trypsin. In our experiments trypsin caused a remarkable decrease in the membrane-bound ATPase activity. This points to a cleavage of peptide bonds at the region of the active centre of the ATPase. In this relationship it is also of interest that, compared with trypsin, subtilisin A and chymotrypsin required concentrations producing a 5–6-fold higher digestion rate to give a similar loss in the ATPase activity.

In view of the facts that trypsin splits peptide linkages involving the carboxylic group of a basic amino acid, that the effect of trypsin was partially counteracted by Mg^{2+}, Na^+ and K^+, and that ATP changed the effect of cations on the enzyme system, one may conclude that arginine or lysine is likely to be involved in the active centre of the $(Na^+ + K^+)$-activated ATPase. This hypothesis is supported by experiments of JEAN AND BADER[17] according to which arginine is possibly located near the phosphorylated site of the $(Na^+ + K^+)$-activated ATPase.

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