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Influence of Protamine and Gangliosides on the Binding of Rubidium by the Na, K-ATPase Solubilized Preparation from Cerebral Microsomes*

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Introduction

It is now generally accepted that sodium and potassium activated membrane adenosine triphosphatase (Na, K-activated ATPase) is closely related to the active transport of monovalent cations[12,15,19]. Difficulties in solubilization of the enzyme, however, has prevented investigations of the more precise mechanism of its action. For this reason, some investigations reporting on the binding of sodium or rubidium by the enzyme have been done using impure enzyme preparations such as membraneous fraction[1,2,3].

Recently, Nakao and his collaborators have reported that they have obtained a highly specific Na, K-activated ATPase from the erythrocyte membrane by sodium iodide (NaI) treatment[10]. We have also obtained a Na, K-ATPase preparation (ATPase preparation) from cerebral microsomes by a slightly modified NaI-treatment[20].

In the present paper, using the NaI-treated microsomal preparations, an attempt is made to find how the binding of rubidium by this ATPase preparation is affected by substances that modify ion movements in intact cerebral tissues. Such substances include protamine, gangliosides, ouabain and others.

Materials and Methods

Preparation of Enzyme: The microsomal fraction was obtained from the grey matter of rabbit cerebral hemispheres as described by Schwartz et al.[13]. To the microsomal suspension was added an equal volume of 1 M-NaCl containing 0.45 mg/mℓ of sodium deoxycholate (DOC). The particle-salt-DOC mixture was immediately centrifuged at 100,000 g for 60 minutes, and the sediment was washed three times with a 0.32 M-sucrose solution containing 5 mM-ethylene diaminetetraacetic acid (EDTA). The DOC-salt-treated microsomes were suspended in cold water to form a concentration of 3 mg protein/mℓ and stored at −20°C. The ATPase preparation purified with NaI was obtained from the DOC-salt-treated microsomes by a slightly modified procedure described by Nakao et al.[10] To 10 mℓ of the DOC-salt-treated microsomal suspension, 10 mℓ of the NaI solution mixture containing 6 mℓ of 6.6 M-NaI (pH was adjusted to 8.0 with tris-HCL buffer), 50 mM-cystein, 5 mM-$MgCl_2$, 3 mM-ATP and 5 mM-EDTA were added slowly with gentle stirring at 0°C. After 10 minutes the solution was diluted with distilled water to a 0.8 M-NaI

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concentration and centrifuged for 20 minutes at 20,000 g. The pellet was washed three times with water containing 5 mM-EDTA by centrifugation and suspended in 5 mℓ of cold water for the enzyme assay.

Assay of ATPase Activity: The standard reaction mixture of 1 mℓ contained (final concentrations): tris-HCl buffer, pH 7.5 (130 mM), $MgCl_2$ (3 mM) and ATP (tris salt) (3 mM). When NaCl (100 mM) was added, the concentration of the tris-HCl buffer was decreased to 30 mM to maintain approximate isosmolarity. After preincubation of the mixture with shaking at 37°C for 5 minutes, the reaction was initiated by adding the ATPase preparations. The reaction was stopped after 10 minutes by adding 1 mℓ of 5% (w/v) trichloroacetic acid. The liberated orthophosphate (Pi) was determined by the method of Lindberg & Ernster[6]. For protein determinations, the biuret method was used[4].

Measurement of Radioactive Rubidium-binding by the ATPase Preparation: The standard reaction mixture of 1 mℓ for measurement of rubidium-binding contained (final concentrations) 130 mM of tris-HCl buffer (pH 7.5), 3 mM of $MgCl_2$, 3 mM of ATP (tris salt) and 30 mM of RbCl containing radioactive rubidium, ^{86}Rb (10,000 c.p.m./mℓ of the reaction mixture). After preincubation of the mixture with shaking at 37°C for 5 minutes, the reaction was initiated by adding the ATPase preparations. In some instances the reaction was initiated by adding ^{86}Rb. The reaction was usually stopped after a 15 second incubation by the method of Millipore filtration[7].

The ultracentifugal method which has been used for the measurement of rubidium- or sodium-binding was found to be completely inadequate for measuring the fast rate of its binding and release under most experimental conditions. In addition, a substantial loss of rubidium occurred in ATP containing systems during the centrifugation period as will be described in the following paragraph.

On the other hand, filtration through millipore filters (type GS or VC with 0.22 to 0.1 μ average pore diameter) is a convenient way to terminate reactions with a sharp end point by the complete removal of the ATPase preparations from the reaction mixture. A set of 25-mm (diameter) Millipore filters were mounted on test tubes with side arms connected to a vacuum line. At appropriate intervals, 1 mℓ of the reaction mixture containing 0.2 to 0.4 mg of the ATPase preparations protein per mℓ were poured on to the filter. The filtration was completed within 2 seconds. The amount of bound rubidium was calculated from the difference between the radioactivity of the filtrates of the ATPase preparation-free incubation medium and of the ATPase preparation-containing samples.

Reagents: Reagents not otherwise specified were of the highest commercial grade. Solutions of ATP (tris salt) were prepared according to the method of Schwartz et al.[13] and stored at a concentration of 30 mM in a deep-freeze. Protamine sulfate (clupeine) was purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Gangliosides were purified Type II (from bovine brain) from Sigma Chemical Co., St Louis, Mo., U.S.A.

Results

ATPase Activity of the ATPase Preparation: The results of a typical purification of the ATPase preparation from cerebral microsomes are shown in Table 1. The ATPase

Table 1 *Solubilization Data of the ATPase Preparation*

Treatment of cerebral microsomes	Specific activity (μ moles Pi/mg protein/hr) in the presence of			Recovery of (per cent)	
	Mg (3mM)	Mg + Na + K (3mM, 100mM, 30mM)	Mg + Na + K+Ouabain (3mM, 100mM, 30mM, 10^{-4}M)	Activity	Protein
DOC-treatment	15.7	61.3	16.2	(100)	(100)
NaI-treatment	7·1	142.2	15.8	62	24

The reaction mixture and the incubating conditions were described in the text.

preparations solubilized with NaI were stimulated by an addition of Na and K to the standard reaction mixture which contained only Mg. The ATPase activity was strongly inhibited by 10^{-4} M ouabain. The ratio of activity of (Mg+Na+K)/Mg on DOC-salt-treated microsomes was about 4. On the other hand, the highest ratio of activity was found in the ATPase preparation (about 20). Phosphate liberation by the ATPase preparation proceeded linearly at least for 60 minutes at 37°C. The pH-activity curve showed a sharp peak at pH 7.5.

The Time Course of Rubidium-binding by the ATPase Preparation: Fig. 1 shows the experimental results on the time course of rubidium-binding in the ATPase preparations. In the absence of ATP and Mg, the rate (corresponds to solid circle) was slow and linear, over the tested interval of one minute after which time a saturation level was reached and maintained.

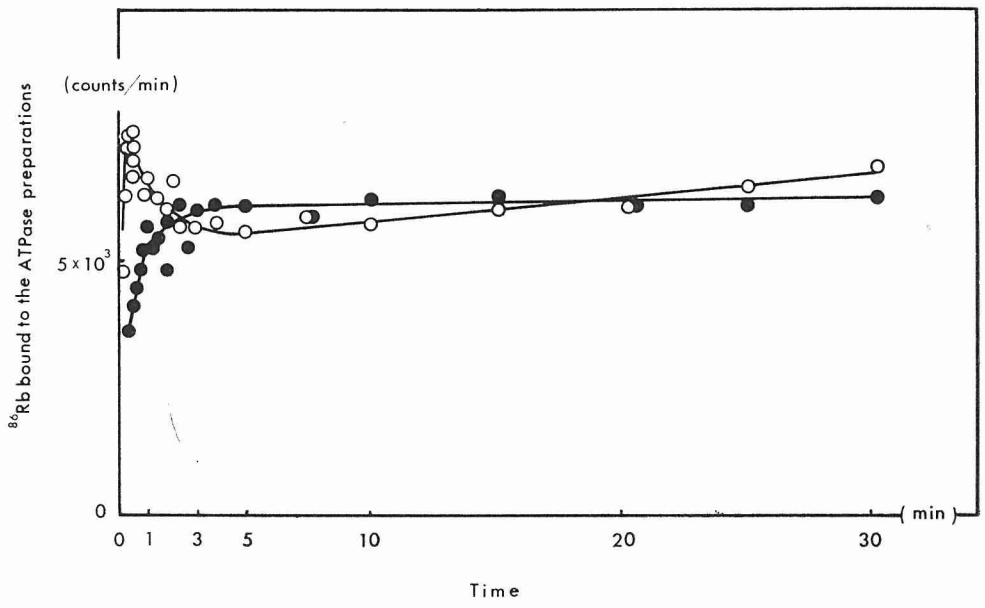

Fig. 1 The time course of Rb-binding by the ATPase preparations.
○——○: with ATP and Mg ●——●: without ATP and Mg
The incubating conditions were described in the text. The reaction was started with the addition of the ATPase preparations and terminated after incubation by filtration through Millipore filters.

In the presence of ATP and Mg, on the other hand, the binding (corresponds to open circle) occured almost instantaneously during the first few seconds after which time a maximum level was reached. A prolonged incubation beyond 30 seconds caused a release of the bound rubidium into the medium. The reaction after two minutes showed no significant difference between the presence or absence of ATP and Mg. After 10 minutes, rebinding was observed.

It was therefore dicided to study the reaction during a second incubation, which permitted work in the rapid initial phase as well as affording reasonably good timing.

Effects of ATP, Ouabain, Monovalent and Divalent Cations on the Rubidium-binding by the ATPase Preparation: Some of the results obtained by changing the components of the medium are compiled in Table 2. When ATP and Mg are omitted from the incubation mixture the binding of rubidium was considerably smaller. The binding was also decreased by ouabain in a concentration of 10^{-4} M. A considerable decrease of the binding was caused by additions of Ca and K. High concentrations of Na had a diluting effect on the binding.

Table 2 *Binding of ^{86}Rb by the ATPase Preparation*

Additions (+) or Omissions (−)	^{86}Rb binding counts/min
None	3,800
−ATP	950
$-Mg^{++}$	1,190
+Ouabain, 10^{-4} M	1,070
$+Ca^{++}$, 1 mM	1,890
$+Na^{+}$, 100 mM	2,600
$+K^{+}$, 30 mM	440

The additions to and omissions from the standard reaction mixture. The standard reaction mixture: ATP (tris salt) (3 mM), $MgCl_3$ (3 mM), RbCl (30 mM) containing ^{86}RbCl and tris-HCl buffer, pH 7.5 (130 mM). Incubation at 37°C for 15 seconds.

Effects of Protamine and Gangliosides on the Rubidium-binding by the ATPase Preparation: Table 3 shows the effects of protamine, gangliosides and cations on the binding of rubidium by the ATPase preparations. Protamine apparently inhibited the binding. Ganglioside itself has no appreciable effect on the rubidium-binding. When the ATPase preparation was incubated in media containing an inhibitory concentration of protamine together with ganglioside, the binding was again normal. The inhibitory effect of protamine was

Table 3 *The Effects of Protamine, Ganglioside and Cations on the Binding of ^{86}Rb by the ATPase Preparation*

Additions to the basic medium	^{86}Rb binding counts/min
None	3,800
Protamine sulphate, 0.10 mg./mℓ.	2,550
Protamine sulphate, 0.25 mg./mℓ.	1,680
Ganglioside, 0.25 mg./mℓ.	4,040
Protamine sulphate, 0.25 mg./mℓ., and ganglioside, 0.25 mg./mℓ.	3,590
Protamine sulphate, 0.25 mg./mℓ., and Rb^{+}, 100 mM	2,140
Protamine sulphate, 0.25 mg./mℓ., and Rb^{+}, 200 mM	2,860
Protamine sulphate, 0.25 mg./mℓ., and Na^{+}, 100 mM	2,250

Conditions otherwise as in the text and Table 2.

also removed by the addition of Rb and Na in excess.

Effect of Protamine on the Release of the Bound Rubidium from the ATPase Preparation: The ATPase preparation was labeled with ^{86}Rb in the standard rection mixture for 10 minutes at 37°C. Thereafter, protamines were added. After incubation of 10 minutes, the bound rubidium by the ATPase preparation was measured by the method of Millipore filtration.

Table 4 shows that low concentrations of protamine did not affect the release of bound rubidium from the ATPase preparation. Higher concentrations of protamine, on the other hand, caused the release of the bound rubidium. From these experiments, it is clear that protamine in a concentration of 0.25 mg/mℓ inhibits the binding of rubidium to the ATPase preparation, but does not affect the release of the bound rubidium from those.

Table 4 *The Effect of Protamine on the Release of the Bound ^{86}Rb from the ATPase Preparation*

Protamine sulphate mg./mℓ.	^{86}Rb binding counts/min
0	2,600
0.10	2,580
0.15	2,350
0.25	2,070
0.5	1,400

The ATPase preparation was labeled with ^{86}Rb in the standard reaction mixture for 10 minutes at 37°C. Thereafter, protamines were added. After incubation of 10 minutes, the bound rubidium by the ATPase preparation was measured by the method of Millipore filtration described in the text.

Effect of Protamine on the ATPase Activity of the ATPase Preparation: The activity of Na, K-activated ATPase after incubation with protamine at 37°C for 15 minutes was only 34% of the control activity, as shown in Table 5. The decrease in activity of ATPase produced by protamine was selectively observed on the Na, K-activated ATPase and it was shown that ATP splitting activity observed with Mg alone was not significantly influenced by protamine. Gangliosides, K and Na thus prevented the inhibitory effect of protamine.

Table 5 *The Effect of Protamine on the Activity of the ATPase Preparation*

Condition	ATPase activity (μ moles Pi/mg protein/hr)						
	Mg 3mM		Mg + Na + K 3mM 100mM 30mM				
	None	Prot.	None	Prot.	Prot.+Gangli.	Prot.+K	Prot.+Na
Incubation at 37° for 15 min.	7.0	6.2	103.7	35.5	97.3	61.1	73.3

Prot. = protamine sulphate, 0.25 mg./mℓ.
Gangi. = ganglioside, 0.25 mg./mℓ.
K = 100 mM
Na = 100 mM

The ATPase activity after incubation with protamine at 37°C for 15 minutes was assayed. The other incubating conditions were described in the text.

Discussions

As described in the accompanying paper[20], the properties of the ATPase preparations

purified with NaI-treatment were quite similar to those of the cerebral microsomes. Therefore, the NaI-procedure developed for erythrocyte membrane was also applicable to cerebral microsomes, resulting in a highly specific and active preparation as shown in Table 1. If the ATPase preparation is a useful material for an intermediate in the active transport, it is considered that the preparation is capable of binding rubidium ions transiently. The results of our experiments described here support this view.

The experimental results on the time course of rubidium-binding by the ATPase preparation indicated that the binding pattern in the presence of ATP and Mg showed a fundamental difference from those in the absence of ATP and Mg (Fig. 1). The reason for this difference is not known at present, However, it is a point of interest that the binding after two minutes showed no significant difference between that in the presence and that in the absence of ATP and Mg.

The characteristics of the mode of the rubidium-binding by the ATPase preparations are similar to the known properties of the Na, K-activated, Mg-dependent and ouabain-sensitive membrane ATPase as shown in Table 2. Namely, the rubidium-binding was dependent on ATP and Mg, and was depressed by ouabain and Ca. ATP dependent rubidium-binding was also decreased by the presence of K. This is in accordance with the general conception that Rb and K are similar electrochemically and have similar affinities for the carrier of K transport. High concentrations of Na had a diluting effect on the binding. This seems to indicate that excess Na can exert an inhibiting effect by competing with Rb for the binding site. These data presented here are compatible with the hypothetical mechanism suggested by Järnefelt[3]. He suggested that a phosphorylated intermediate formed in the hydrolysis of ATP binds Na in the presence of ATP and Mg, and postulated that the intermediate may act as a transient "carrier" in an active transport mechanism.

Recently, protamine and other basic proteins have been reported to alter the properties of surface membranes of brain slices[8,9,18]. In addition to this, several authors have described the effect of protamine and gangliosides on the activity of ATPase in microsomal preparations[13,14,18]. Therefore, it seems a matter of interest to examine the effects of protamine and gangliosides on the binding of rubidium by ATPase preparations.

Protamine apparently inhibited the rubidium-binding by the ATPase preparations as shown in Table 3. The inhibitory effect of protaimne was removed by the addition of gangliosides and excess cations. Low concentrations of protamine did not affect the release of bound rubidium from the ATPase preparations. Higher concentrations of protamine, however, caused the release of the bound rubidium as shown in Table 4.

In order to understand the rubidium-binding by the ATPase preparation further, an attempt has been made to find how the ATPase activity is affected by protamine and others. Pretreatment of the ATPase preparation with protamine at 37°C for 15 minutes resulted in a loss of Na, K-activated ATPase activity, though the activity of Mg-ATPase was not influenced. Gangliosides, Na and K prevented this inhibitory effect of protamine as shown in Table 5. The selective inactivation of Na, K-activated ATPase and the suppression of the rubidium-binding caused by protamine seemed to be antagonized by gangliosides and cations. Under our experimental conditions, the inhibition of the

rubidium binding by protamine may be due largely to the inactivation of Na, K-activated ATPase. It is possible to conclude from the results described here that the reaction involving the ATPase and the rubidium-binding seem to stem from the same phenomenon.

Furthermore, the present findings with the substances that affect the rubidium-binding and the ATPase activity suggest an involvement of basic and acidic centers in the enzyme action.

Now, McIlwain et al,[9] proposed a mechanism in which the ganglioside function exerted itself at membrane pores of the type envisaged by Danielli and others, in association with the Na, K-activated ATPase. Accordingly protamine could inhibit cation transport at such pores by combining with acidic groups at its mouth, hence interrupting a sequence of negatively charged adsorption sites and, at the same time added ganglioside could restore the acidic environment by masking the basic groups of protamine while still leaving it attached to the membrane. The present studies provide experimental evidence for McIlwain's statement.

It has been also reported that protamine seems to act on phospholipids[5]. On the other hand, there are many reports to show that phospholipids play an important role in the activity of Na, K-activated ATPase[11,16,17]. Therefore, it seems reasonable that the rubidium-binding and the Na, K-activated ATPase are sensitive to protamine treatment. Further experimentation is necessary to explain fully this interesting investigation.

Summary

1) A highly specific and active Na, K-ATPase preparation was obtained from cerebral microsomes by a slightly modified NaI-treatment. Using the ATPase preparations, the effects of protamine and gangliosides on the binding of rubidium and the activity of Na, K-activated ATPase were examined.

2) The results on the time course of the rubidium-binding by the ATPase preparation indicated that the binding pattern in the presence of ATP and Mg showed a fundamental difference from those in the absence of ATP and Mg.

3) The rubidium-binding by the ATPase preparation was dependent on ATP and Mg. The ATP dependent rubidium-binding was inhibited by ouabain, Ca and K. High concentrations of Na also decreased the binding.

4) The ATP dependent rubidium-binding was apparently inhibited by protamine. The inhibitory effect of protamine was removed by the addition of gangliosides and excess cations.

5) Low concentrations of protamine did not affect the release of bound rubidium from the ATPase preparation. Higher concentrations of protamine caused release of the bound rubidium.

6) The Na, K-activated ATPase activity of the ATPase preparation was selectively inhibited by protamine, though the activity of Mg-ATPase was not influenced. These effects of protamine on the Na, K-activated ATPase activity were antagonized by the addition of gangliosides and cations.

7) The actions of protamine and gangliosides on the binding of rubidium and the

Na, K-activated ATPase were discussed on the basis of these results.

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