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K^+-DEPENDENT PHOSPHATASE ACTIVITY OBSERVED IN THE PRESENCE OF BOTH ADENOSINE TRIPHOSPHATE AND Na^+

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SUMMARY

1. Addition of both ATP and Na^+ caused a remarkable increase in the activity of the K^+-dependent phosphatase at low concentrations of K^+, although addition of either ATP or Na^+ alone was inhibitory. The apparent K_m for K^+ decreased from 2 mM to p.2 mM on addition of both substances. The v_{max} was depressed by them.

2. CTP, ITP, and ADP showed effects similar to ATP in the presence of Na^+. The optimal concentrations of ATP and ADP were observed to be 10^{-4} M and $2 \cdot 10^{-4}$ M, but that of CTP and ITP was 10^{-3} M. AMP, 3',5'-cyclic AMP, CDP, UTP, and GTP had no significant stimulatory effect.

3. Replacement of Na^+ with other monovalent cations, such as Rb^+, NH_4^+, Cs^+, or Li^+, caused the stimulatory effect of ATP to disappear.

4. The K^+-dependent phosphatase became heat labile on addition of K^+, but was stabilized by further addition of Na^+ and ATP.

5. Ouabain inhibited the K^+-dependent phosphatase activity more in the presence of ATP and Na^+ than in their absence.

6. The K^+-dependent phosphatase activity was less sensitive to treatment with F^- in the presence of ATP and Na^+ than in their absence.

7. From these results it was concluded that the affinity of the K^+-dependent phosphatase for K^+ was increased by addition of ATP and Na^+, and the properties came closer to those of the (Na^+-K^+)-ATPase.

INTRODUCTION

It is generally considered that the reaction catalyzed by the (Na^+-K^+)-dependent ATPase consists of two parts[1-4]. The first is a Na^+-dependent phosphorylation of the enzyme and the second is K^+-dependent dephosphorylation of the phosphorylated intermediate. The phosphatase activity which appears in the (Na^+-K^+)-ATPase preparation in the presence of Mg^{2+} and K^+ is known to be similar in many respects to the activity in the latter half of the (Na^+-K^+)-dependent ATPase reaction[5-11]. Thus it is possible that the K^+-dependent hydrolysis of phosphate compounds may be catalyzed by the (Na^+-K^+)-ATPase.

The properties of the K^+-dependent phosphatase have been examined and it is known that both ATP and Na^+ inhibit the enzyme[5,8–11]. However, it was found in our laboratory that the coexistence of Na^+ and ATP activated the K^+-dependent phosphatase under certain conditions[12]. In this paper, the properties of the K^+-dependent phosphatase, which is active in the presence of ATP and Na^+, are described and compared with those of the (Na^+-K^+)-ATPase.

MATERIALS AND METHODS

Enzyme preparation

Adult guinea pigs were sacrificed by decapitation, and the brains were immediately taken out and homogenized in 0.32 M sucrose in a Teflon homogenizer. After removal of the large granular fraction by centrifugation at 10 000 × *g* for 15 min, the light granular fraction was spun down at 100 000 × *g* for 60 min. The pellet was suspended in distilled water to give 10–20 mg protein per ml and treated with NaI by the method of NAKAO *et al.*[13], with the following slight modifications. 800 ml of the suspension were mixed with 40 ml of a solution consisting of 0.14 M Tris–HCl buffer (pH 7.4), 12 mM Na_2ATP and 15 mM cysteine, and then 20 ml of 4.2 M NaI solution was added with slow stirring. The mixture was allowed to stand for 15 min at 0° and then centrifuged at 105 000 × *g* for 30 min. The pellet was resuspended in 40 ml of cold water, and 7 ml of 0.4 M Tris–HCl buffer (pH 7.4), 7 ml of 50 mM $MgCl_2$, 7 ml of 50 mM cysteine, 7 ml of 40 mM Na_2ATP, and 2 ml of 100 mM EDTA were added. 70 ml of 6 M NaI were then added drop-wise with stirring. After 15 min the mixture was centrifuged at 105 000 × *g* for 40 min, and the material floating on the surface of the tube was collected, washed three times with water by centrifugation, and used as the enzyme preparation. All procedures described above were carried out at 0°. In the preparations thus obtained, K^+-dependent phosphatase activity (μmoles *p*-nitrophenol liberated per mg protein per h at 37°) with 5 mM of K^+ was 3–4, the ratio of activities (Mg^{2+}-K^+/Mg^{2+}) was 14–15, (Na^+-K^+)-ATPase activity (μmoles P_i liberated per mg protein per h at 37°) was 30–80, and the ratio of ATPase activities (Mg^{2+}-Na^+-K^+/Mg^{2+}) was 6–7.

Determination of K^+-dependent phosphatase activity

The basic medium for determination of phosphatase activity contained 40 mM Tris–HCl buffer (pH 7.8), 5 mM $MgCl_2$, 5 mM of *p*-nitrophenylphosphate as substrate and various concentrations of KCl. Incubation was carried out at 37° for 15 min. At the end of the incubation, 2 ml of 0.5 M NaOH were added to 2 ml of the reaction mixture. The amount of *p*-nitrophenol liberated was estimated by measuring the absorbance of the clear supernatant at 410 mμ. K^+-dependent phosphatase activity was calculated by subtracting the activity in K^+-free medium from that in medium containing K^+.

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

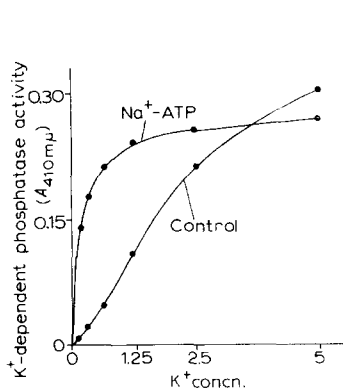
(Na^+-K^+)-ATPase activity was determined in medium containing 40 mM of Tris buffer (pH 7.4), 2 mM of $MgCl_2$, 2 mM of ATP with or without 100 mM of NaCl and 5 mM of KCl. Incubation was carried out at 37° for 30 min. The reaction was stopped by addition of trichloroacetic acid to 10%. The amount of P_i in the acid extract was

estimated. The activity of (Na^+-K^+)-ATPase was taken as the difference between the activities with or without Na^+ and K^+.

RESULTS

Effects of ATP and Na^+ on K^+-dependent phosphatase at low concentrations of K^+

It was reported that addition of ATP or Na^+ alone had an inhibitory effect on K^+-dependent phosphatase[5,8-11]. However, a stimulatory effect of addition of both ATP and Na^+ was found at low concentrations of K^+, as shown in Fig. 1. It seems that the affinity of the enzyme for K^+ was increased by the presence of ATP and Na^+.



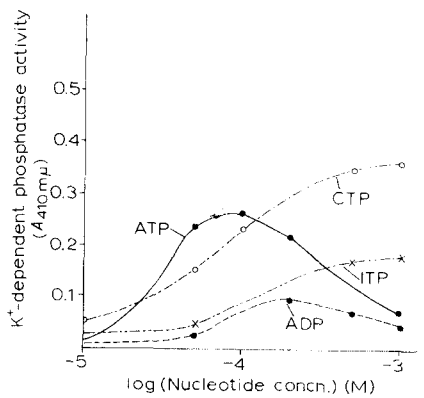


Fig. 1. K^+-dependent phosphatase activities in the presence and absence of ATP and Na^+. Basic medium; 40 mM Tris-HCl buffer (pH 7.8), 5 mM $MgCl_2$, 5 mM *p*-nitrophenylphosphate, 20 mM NaCl, 0.1 mM ATP.

Fig. 2. Stimulative effect of nucleotides on K^+-dependent phosphatase activities in the presence of Na^+. Basic medium; 40 mM Tris-HCl buffer (pH 7.8), 5 mM *p*-nitrophenylphosphate, 5 mM $MgCl_2$, 0.5 mM KCl, 20 mM NaCl.

The apparent K_m for K^+ (the concentration of K^+ giving 50% of the maximum activity) was decreased from about 2 mM in the absence of ATP and Na^+ to 0.2 mM in their presence. The K_m for *p*-nitrophenylphosphate was not affected by the presence of ATP and Na^+. The maximum velocity, v_{max}, was depressed by addition of ATP and Na^+.

Another interesting finding was that a sigmoid curve was obtained for K^+ in the absence of ATP and Na^+ as shown in Figs. 1 and 3.

Nucleotide specificity for the stimulatory effect

The nucleotide specificity for the stimulatory effect in the presence of Na^+ on K^+-dependent phosphatase was examined using various nucleotides in place of ATP. ADP, AMP, 3',5'-cyclic AMP, CTP, CMP, UTP, GTP, and ITP were tested. Among these, ADP, CTP, and ITP were significantly effective, as shown in Fig. 2. The optimal concentrations for ATP and ADP were 10^{-4} M and $2 \cdot 10^{-4}$ M, respectively, though those of CTP and ITP were found to be 10^{-3} M. Next, the effects of CTP and ADP were examined at various concentrations of K^+. As shown in Fig. 3, the influ-

ence of addition of CTP or ADP was very similar to that of addition of ATP. That is, the presence of both nucleotide and Na^+ results in an increase in the affinity of the enzyme for K^+.

Time course of activation of K-dependent phosphatase by ADP and Na^+

Fig. 4 shows that addition of ADP caused an immediate increase in activity without a lag phase, as in the case of ATP addition. If we consider this result together with the finding that the optimal concentration of ADP was not so different from that of ATP, it seems unlikely that ADP shows its effect after conversion to ATP.

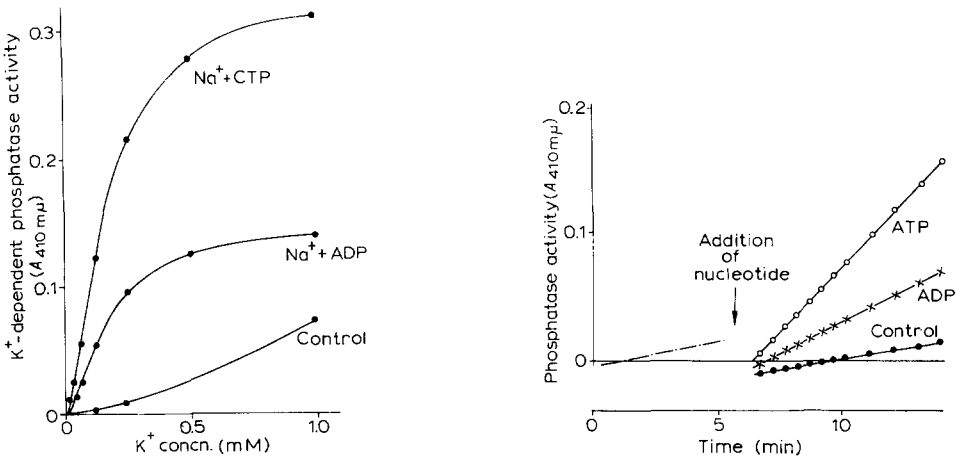


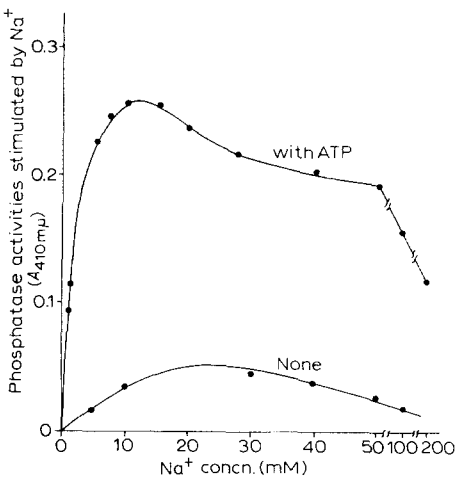
Fig. 3. Effects of CTP, ADP, and Na^+ on K^+-dependent phosphatase activity. Basic medium: 40 mM Tris–HCl buffer (pH 7.8), 5 mM $MgCl_2$, 5 mM *p*-nitrophenylphosphate. 20 mM NaCl, 0.1 mM ADP, 1 mM CTP.

Fig. 4. Time course of increase in phosphatase activity on addition of nucleotide. At the time shown by an arrow, 0.3 ml of 1 mM nucleotide solution was added to 3 ml of reaction mixture containing 40 mM Tris–HCl buffer (pH 7.8), 5 mM $MgCl_2$, 20 mM NaCl, 0.5 mM KCl, 5 mM *p*-nitrophenylphosphate, and enzyme preparation. The reactions were carried out at 30°.

Effect of monovalent cations on activity of K^+-dependent phosphatase in the presence of ATP

K^+-dependent phosphatase activities with and without ATP were estimated in the presence of Na^+, Rb^+, NH_4^+, Cs^+, or Li^+. In the presence of Na^+, ATP caused an increase in activity of K^+-dependent phosphatase, as shown in Fig. 5. The optimal concentration of Na^+ was 10–20 mM with 0.5 mM of K^+. With higher concentrations of K^+, however, there was an increase in the optimal concentration of Na^+ for maximal stimulation by ATP.

When Rb^+, NH_4^+, or Cs^+ was used instead of Na^+, phosphatase activity increased, as shown in Fig. 6. The results indicate that Rb^+, NH_4^+, and Cs^+ are effective substitutes for K^+, reconfirming the results reported in previous papers[5,8,9]. However, in these cases ATP did not stimulate activity. Accordingly, it seems that Na^+ is required specifically to obtain the stimulatory effect of the nucleotides on the K^+-dependent phosphatase.



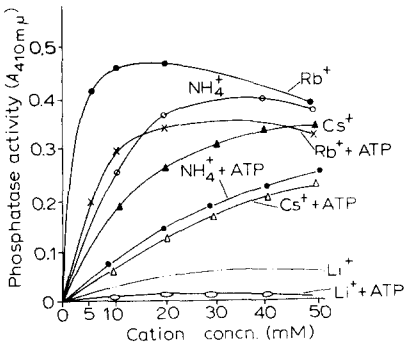


Fig. 5. Influence of Na^+ on nucleotide stimulation of K^+-dependent phosphatase by ATP. Basic medium; 40 mM Tris–HCl buffer (pH 7.8), 5 mM $MgCl_2$, 0.5 mM KCl, 5 mM *p*-nitrophenylphosphate, ATP added 0.1 mM.

Fig. 6. Effect of monovalent cations on phosphatase activity in the presence and absence of ATP.

Effects of cations and ATP on heat denaturation of K^+-dependent phosphatase

The above findings indicate that Na^+ and ATP strongly modify the activity of the K^+-dependent phosphatase. Next, the effects of K^+, Na^+ and ATP on heat denaturation of the enzyme were examined to see whether these ions caused a structural change in the enzyme. As shown in Table I, addition of K^+ decreased the heat stability of the enzyme, while Na^+ and ATP increased it. These results seem to support

TABLE I

EFFECTS OF CATIONS AND ATP ON HEAT DENATURATION OF K^+-DEPENDENT PHOSPHATASE AND (Na^+-K^+)-ATPase

The enzyme preparation was heated at 60° for 15 min in 40 mM Tris–HCl buffer (pH 7.4) containing cations or ATP as listed below. Then, ice-cold water was added and mixtures were centrifuged at 12 000 × *g* for 20 min. The pellet was suspended in water, and K^+-dependent phosphatase and (Na^+-K^+)-ATPase activities were estimated. (See MATERIALS AND METHODS.)

*Conditions for pretreatment**	*Enzyme activities (%)*	
	K^+-dependent phosphatase	*(Na^+-K^+)-ATPase*
Untreated	100	100
Treated with:		
Mg^{2+} (5)	40.8	43.7
Mg^{2+} (5), K^+ (1)	36.1	41.7
Mg^{2+} (5), Na^+ (20)	44.4	51.7
Mg^{2+} (5), K^+ (1), Na^+ (20)	47.2	49.4
Mg^{2+} (5), K^+ (1), Na^+ (20), ATP (0.1)	65.4	62.7

* Concentrations of added compounds in mM are shown in parentheses.

the consideration that Na^+ and ATP cause a structural change in the enzyme, whereby the K^+-dependent reaction becomes marked.

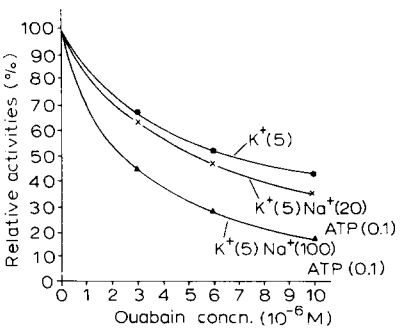
Change in ouabain sensitivity of K^+-dependent phosphatase by ATP and Na^+

Ouabain is known to be a typical inhibitor of the (Na^+-K^+)-dependent ATPase, and it also inhibits K^+-dependent phosphatase. However, K^+-dependent phosphatase was less sensitive to ouabain than (Na^+-K^+)-dependent ATPase when *p*-nitrophenylphosphate was used as substrate. This difference is contrary to the idea that the two activities are catalyzed by the same enzyme molecule.

As shown in Fig. 6, however, the sensitivity of the K^+-dependent *p*-nitrophenylphosphate hydrolyzing activity to ouabain increased on addition of both ATP and Na^+, especially when 100 mM of Na^+ were used, showing a similarity to the activity of the K^+-dependent acetylphosphatase[10]. This means that the properties of K^+-dependent phosphatase become more similar to those of the (Na^+-K^+)-dependent ATPase on addition of Na^+ and ATP. In this experiment the K^+ concentration was fixed at 5 mM, because it has been reported that the inhibition of the K^+-dependent phosphatase by ouabain was nearly competitive with K^+ (ref. 5).

Effect of pretreatment with F^- on the activities of the K^+-dependent phosphatase in the presence and absence of Na^+ and ATP

It has been reported that pretreatment of the (Na^+-K^+)-ATPase preparation with F^- caused greater inactivation of the K^+-dependent phosphatase than of the (Na^+-K^+)-ATPase[14]. Our findings, however, indicate that there may be two different



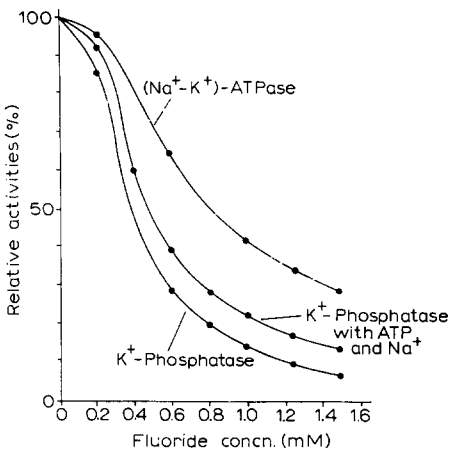


Fig. 7. Change in ouabain sensitivity of K^+-dependent phosphatase in the presence of Na^+ and ATP. Experimental procedure described in MATERIALS AND METHODS. Concentrations of added compounds in mM are shown in parentheses.

Fig. 8. Effect of pretreatment with F^- on activities of (Na^+-K^+)-ATPase and K^+-dependent phosphatase in the presence and absence of ATP and Na^+. Enzyme preparation suspended in 2 ml of solutions containing 40 mM Tris–HCl buffer (pH 7.8), 5 mM $MgCl_2$, 5 mM K^+ and various concentrations of F^- were incubated at 37° for 10 min. 5 ml of ice-cold water were added to stop the reaction, and mixtures were immediately centrifuged at 12 000 × *g* for 20 min. The pellet was rewashed and suspended in water for use as pretreated enzyme. The three enzyme activities were then estimated.

types of K^+-dependent phosphatase activity which are distinguishable in the presence and absence of ATP and Na^+. Accordingly, the activities of the two different types of K^+-dependent phosphatase were examined in a preparation which had been pretreated with F^-. K^+-dependent phosphatase activity was less affected by treatment with F^- in the presence of ATP and Na^+ than in their absence. The results also seem to support the idea that K^+-dependent phosphatase was more closely related to (Na^+-K^+)-ATPase in the presence of ATP and Na^+ than in their absence.

DISCUSSION

It is widely known that (Na^+-K^+)-ATPase and K^+-dependent phosphatase have similar properties in many respects, and it has been suggested that the latter step, dephosphorylation of the phosphorylated intermediate of (Na^+-K^+)-ATPase, and K^+-dependent *p*-nitrophenylphosphate hydrolyzing activity might be catalyzed by the same enzyme. However, there are some differences between the two reactions. These include: (1) The K_m of K^+-dependent phosphatase is higher than that of (Na^+-K^+)-ATPase. (2) Ouabain inhibits (Na^+-K^+)-ATPase more than K^+-dependent phosphatase. (3) On treatment with F^-, K^+-dependent phosphatase is inactivated more than (Na^+-K^+)-ATPase.

These differences are diminished by using acylphosphate as substrate instead of *p*-nitrophenylphosphate, as previously reported[6,7,9–11]. Furthermore, as shown in this paper, K^+-dependent phosphatase activity, estimated in the presence of both Na^+ and ATP, comes closer to that of (Na^+-K^+)-ATPase than in medium containing only Mg^{2+} and K^+. Thus on considering the properties of K^+-dependent phosphatase in the presence of Na^+ and ATP, it appears more likely that its activity may represent the latter dephosphorylation process of the phosphorylated intermediate of the (Na^+-K^+)-ATPase reaction.

The present results show that Na^+ is specific for stimulation of K^+-dependent phosphatase activity by ATP. This is interesting in connection with the well-known fact that Na^+ is absolutely necessary for the activity of (Na^+-K^+)-ATPase, although K^+ can be replaced by ions such as Rb^+, NH_4^+, or Cs^+ (refs. 15–17). In the presence of Na^+, ATP, ADP, CTP, and ITP had stimulatory effects, as shown in Fig. 2. The observation that ATP and CTP had strong stimulatory effects, together with the reports that they are effective substrates of partially purified (Na^+-K^+)-ATPase treated with NaI (refs. 18, 19), again indicate an intimate relation between the two enzyme activities.

The idea that the enzyme may have a different structure in the presence of Na^+ and ATP is supported by the results on heat denaturation indicated in Table I. Similar findings on the difference in stability of the (Na^+-K^+)-ATPase preparation in the presence and absence of Na^+ and ATP were reported for the inactivations by F^- (ref. 14), DFP (ref. 20), Be^{2+} (ref. 21), and *N*-ethylmaleimide[22]. Recently, the binding of a cardiac glycoside to (Na^+-K^+)-ATPase preparation was reported to be due to a structural change caused by Na^+ and nucleotide[23]. Our finding that the affinity of the enzyme for K^+ was increased by the presence of Na^+ and nucleotide may be relevant to the report[23], because it is generally considered that the site of action of ouabain and K^+ is the same.

There are two possible ways in which the presence of Na^+ and nucleotide might

change the structure of the enzyme. First, in regard to the state of the enzyme, a phosphorylated intermediate of the enzyme may be formed. It seems likely that the formation of a phosphorylated intermediate would increase the affinity of the enzyme for K^+. However, ADP caused a similar stimulatory effect on the K^+-dependent phosphatase without any lag phase and the optimum was at 10^{-4} M, which is almost the same as that of ATP. ITP had a similar effect. ADP and ITP were reported to be hydrolyzed only slightly or insignificantly by the partially purified preparation of (Na^+-K^+)-ATPase[18,19]. The other possibility is that the nucleotides have a direct influence on the enzyme as effectors by binding with the enzyme without phosphorylation. In this case, our findings may indicate that Na^+ not only stimulates the first phosphorylation process of the (Na^+-K^+)-ATPase reaction, but also affects the latter dephosphorylation step, and ATP or ADP also serves as an effector of the dephosphorylation process of the (Na^+-K^+)-ATPase reaction, perhaps by changing the structure of the enzyme.

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