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# ADP BINDING TO $(Na^+ + K^+)$-ACTIVATED ATPase

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## SUMMARY

[$^{14}$C]ADP binding to EDTA-washed ox brain cell membranes was increased by $Na^+$, but decreased by $K^+$, $Mg^{2+}$ and $Ca^{2+}$. $Na^+$ abolished the effect of $K^+$ on ADP binding by a competitive mechanism, but could not reverse the inhibitory action of $Mg^{2+}$ and $Ca^{2+}$. It is concluded that the cation-induced changes in ADP binding reflect properties of $(Na^+ + K^+)$-activated ATPase.

## INTRODUCTION

$(Na^+ + K^+)$-activated ATPase (EC 3.6.1.3) is assumed to undergo conformational changes during active $Na^+$ transport[1,2]. A conformational state $E_1$ is assumed to be formed at low $Mg^{2+}$ concentrations. It is characterized by high affinity for ATP and $Na^+$, but low affinity for the specific inhibitor ouabain. The conformational state $E_2$, which is formed at high $Mg^{2+}$ concentrations, is characterized by a high affinity for ouabain but no or low affinity for ATP[3]. This hypothesis is supported by many different kinds of experiments[4–8]. The concept of cation-induced conformational changes is also supported by studies on the binding of [$\gamma$-$^{32}$P]ATP to $(Na^+ + K^+)$-activated ATPase[9,10]. In the presence of $Mg^{2+}$, ATP is hydrolysed so that it is difficult to assay the effect of $Mg^{2+}$ on nucleotide binding. This obstacle may be overcome by the use of radioactive [$^{14}$C]ADP. ADP is a competitive inhibitor of $(Na^+ + K^+)$-activated ATPase[11] and a cosubstrate of the $Na^+$-dependent ATP–ADP transphosphorylation reaction[4], the latter reaction being a partial reaction of the overall reaction[4]. In this paper we report the effects of mono- and divalent cations on ADP binding to ox brain cell membranes.

## METHODS AND MATERIALS

$(Na^+ + K^+)$-activated ATPase from ox brain[12] was converted to the $E_1$ form[3] by washing twice with 10 mM Tris–EDTA and subsequently with distilled water.

[$^{14}$C]ADP binding to $(Na^+ + K^+)$-activated ATPase from ox brain was measured by a rapid mixing centrifugation method[10]. After incubation of 0.8–2.0 mg enzyme protein for 0.5 to 3 min at 0 °C with 50 mM Tris (pH 7.4), 93 nM [$^{14}$C]ADP and the additions mentioned in the figures in a total volume of 1 ml, the membranes were spun down at $70000 \times g$. After careful removal of any remaining incubation

mixture from the walls of the polypropylene centrifuge tubes, the sediment was dissolved in 1 ml 1 M NaOH at 50 °C. The solubilized protein was neutralized with conc. HCl, dissolved in a scintillation solution containing Triton X-100[9] and counted in a TriCarb 3375. Each experimental point was done in duplicate or triplicate. All results were corrected for unspecific ADP binding by subtractings the value obtained in the presence of 10 mM unlabelled ADP.

[$^{14}$C]ADP was from Schwarz-Mann, Orangeburg, N.Y., U.S.A. (lithium salt, spec. act. 288–359 Ci/mole) or from Amersham-Buchler, Braunschweig, Germany (ammonium salt, spec. act. 535 Ci/mole).

## RESULTS AND DISCUSSION

### *Differential action of $Na^+$ and $K^+$ on ADP binding*

Incubation of ox brain cell membranes with increasing concentrations of $Na^+$ results in an increase of [$^{14}$C]ADP binding (Fig. 1A). High concentrations of $Na^+$ inhibited ADP binding. This finding agrees with the demonstration of an inhibitory action of high $Na^+$ concentrations in the $Na^+$-activated but $K^+$-inhibited hydrolysis of UTP by ($Na^+ + K^+$)-activated ATPase from electric eel microsomes[13]. The inhibitory effect was most pronounced at 80 mM (not shown). ADP binding to ox brain cell membranes was reduced by increasing $K^+$ concentrations in the absence of $Na^+$ (Fig. 1B). This effect is caused by a decreased affinity of the enzyme to ADP as it is evident by the increase of the apparent dissociation constant of the enzyme–ADP complex in the presence of $K^+$ (Fig. 2). A similar pattern has been reported for the effect of $K^+$ on the enzyme–ATP complex[9,10].

The apparent dissociation constant of the enzyme–ADP complex in the absence of $K^+$ but presence of 5 mM $Na^+$ calculated from the slope of the Scatchard plot (Fig. 2) was 0.34 $\mu$M. This value is close to that calculated by Jensen and Nørby[14] from competition studies with ADP on the binding of [$\gamma$-$^{32}$P]ATP on the enzyme. Increasing concentrations of $K^+$ raised the dissociation constants of the enzyme–ADP complex to 0.54 $\mu$M (0.5 mM $K^+$) and 6.6 $\mu$M (5 mM $K^+$), respectively. In agreement with studies on ATP binding to ($Na^+ + K^+$)-activated ATPase[10], increasing concentrations of $Na^+$ abolished the effect of $K^+$ on ADP binding by a competitive mechanism (Fig. 1A).

The above-reported similarities between the binding of ADP and ATP[9,10,14] indicate that the capability of ox brain membranes to bind ADP reflects a property of ($Na^+ + K^+$)-activated ATPase.

### *Inhibition of ADP binding by $Mg^{2+}$ and $Ca^{2+}$*

In agreement with the hypothesis that $Mg^{2+}$ induces a conformational state of the enzyme with a low affinity for the substrate[3], $Mg^{2+}$ reduced ADP binding to ox brain cell membranes (Fig. 3A). Just as with $K^+$, reduction of ADP binding in the presence of $Mg^{2+}$ was caused by an increase in the dissociation constant of the enzyme–ADP complex (not shown). The effect of $Mg^{2+}$ in decreasing ADP binding to ox brain cell membranes could be enhanced further by $K^+$, when the action of $K^+$ was studied at suboptimal $Mg^{2+}$ concentrations (0.1 mM). However, in contrast to the counteraction of $Na^+$ on the decreased nucleotide binding in the presence of $K^+$ (Fig. 1A), $Na^+$ could only partially overcome the inhibitory effect of $Mg^{2+}$

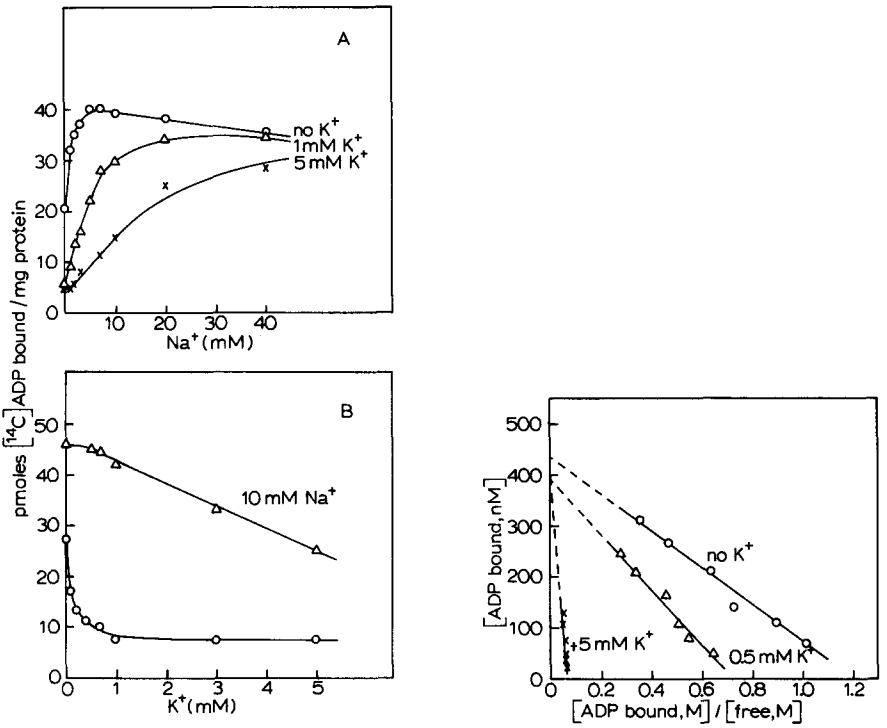


Fig. 1. Activation of [$^{14}$C]ADP binding to ox brain cell membranes by $Na^+$. (A) Competitive action of $K^+$ on the $Na^+$-induced enhancement of ADP binding. The incubation medium contained in addition: no $K^+$ (○—○); 1 mM $K^+$ (△—△); 5 mM $K^+$(×—×). (B) Decrease of ADP binding by increasing $K^+$ concentrations. Binding in the presence of 10 mM $Na^+$, (△—△); no additions (○—○).

Fig. 2. Scatchard plot of the binding of ADP to ox brain cell membranes with 5 mM $Na^+$ in the absence and presence of $K^+$. Specific activity of the enzyme preparation: 3.75 $\mu$moles·mg$^{-1}$·min$^{-1}$. Binding in the presence of 5 mM $K^+$ (×—×); 0.5 mM $K^+$ (△—△); no additions (○—○). From the slope of the line in the absence of $K^+$ the dissociation constant $K_D$ of the enzyme–ADP complex was calculated to be 0.34 $\mu$M. $K_D$ in the presence of 0.5 mM $K^+$ is 0.54 $\mu$M, in the presence of 5 mM $K^+$ 6.6 $\mu$M.

on ADP binding (Fig. 3B). This effect of $Na^+$ however, could only be demonstrated at low $Mg^{2+}$ concentrations (0.2 mM).

In the light of the results obtained with $Mg^{2+}$, it seemed to be worthwile to study the effect of $Ca^{2+}$ on the capability of ox brain cell membranes to bind ADP. $Ca^{2+}$ inhibits $(Na^+ + K^+)$-activated ATPase at relatively low concentrations[15,16]. As shown in Fig. 4, $Ca^{2+}$ decreases the ability of the enzyme to bind ADP. Because ADP is bound in the absence of any divalent cations, it could be argued that the decrease in ADP binding is caused by the formation of a $Ca^{2+}$–ADP complex. This assumption, however, is not justified because at a concentration of 50 $\mu$M $Ca^{2+}$, no effect of $Ca^{2+}$ on ADP binding is seen (Fig. 4A). At this $Ca^{2+}$ concentrations all free ADP should be transformed into the $Ca^{2+}$–ADP complex ($Ca^{2+}$:ADP ratio = 573). Furthermore, in the presence of 5 mM $Na^+$ 100 $\mu$M $Ca^{2+}$ even slightly stimulated ADP binding

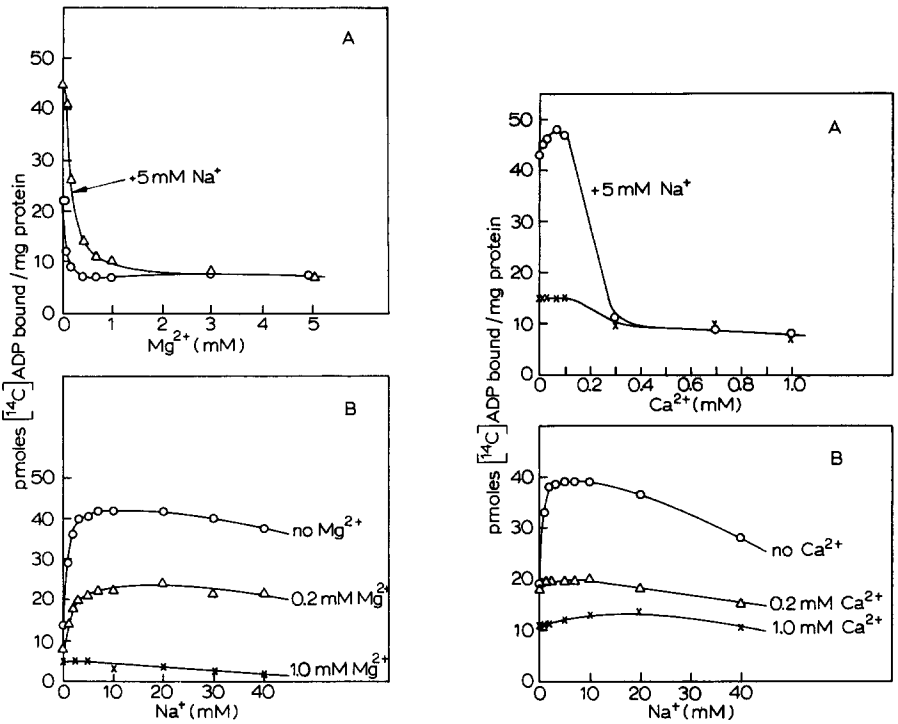


Fig. 3. Effect of $Mg^{2+}$ on ADP binding to ox brain cell membranes. (A) Demonstration of the inhibitory action of increasing $Mg^{2+}$ concentrations. Assay in the presence of 5 mM $Na^+$ (△—△); no additions (○—○). (B) Effect of $Mg^{2+}$ on the $Na^+$-induced enhancement of ADP binding. The incubation medium contained in addition: no $Mg^{2+}$ (○—○); 0.2 mM $Mg^{2+}$ (△—△); 1 mM $Mg^{2+}$ (×—×).

Fig. 4. Effect of $Ca^{2+}$ on ADP binding to ox brain cell membranes. (A) Demonstration of the inhibitory action of increasing $Ca^{2+}$ concentrations. Assay in the presence of 5 mM $Na^+$ (○—○); no additions (×—×). (B) Absence of a counteracting effect of $Na^+$ on $Ca^{2+}$ inhibition. The incubation medium contained in addition: no $Ca^{2+}$ (○—○); 0.2 mM $Ca^{2+}$ (△—△); 1 mM $Ca^{2+}$ (×—×).

(Fig. 4A); this effect was not shown by $Mg^{2+}$ in the presence of 5 mM $Na^+$ (Fig. 3A). The results of the study on ADP binding to ox brain cell membranes at low $Ca^{2+}$ concentration support the conclusions of Epstein and Whittam[16], drawn from their kinetic studies on the $Ca^{2+}$ inhibition of $(Na^+ + K^+)$-activated ATPase: The $Ca^{2+}$–nucleotide complex as well as the $Mg^{2+}$–nucleotide complex bind to the active site of the enzyme. Higher concentrations of $Ca^{2+}$ (above 0.2 mM) affect ADP binding to the enzyme probably in a way similar to $Mg^{2+}$: The decrease of ADP binding in the presence of $Ca^{2+}$ was caused by an increase in the dissociation constant of the enzyme–ADP complex. Additional similarities between the action of $Ca^{2+}$ and $Mg^{2+}$ are evident from a study on the effect of $Na^+$ on ADP binding in the presence of $Ca^{2+}$ (Fig. 4B): $Na^+$ could not abolish the inhibitory action of $Ca^{2+}$, a finding which is in contrast to the report of Portius and Repke[17] who found a reversal of $Ca^{2+}$ inhibition of $(Na^+ + K^+)$-activated ATPase by $Na^+$. From the similarities

in the action of $Mg^{2+}$ and $Ca^{2+}$ it appears that the inhibitory action of $Ca^{2+}$ on $(Na^+ + K^+)$-activated ATPase could at least be partially caused by an alteration of the enzyme structure. This latter conclusion would be in agreement with the finding that $Ca^{2+}$ promotes ouabain binding to enzyme preparations from pig and dog heart[18,19].

The data presented in this report show that ADP binding to $(Na^+ + K^+)$-activated ATPase is strongly influenced by mono- and divalent cations. It is to be expected that more intensive studies on ADP binding will give further insight into the properties of the enzyme.

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