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EFFECTS OF ALKALI METAL CATIONS ON PHOSPHO-ENZYME LEVELS AND [^{3}H]OUABAIN BINDING TO ($Na^+ + K^+$)-ATPase

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Summary

The effects of several alkali metal cations on the relationship between steady state phospho-enzyme levels and initial velocity and equilibrium levels of [^{3}H]-ouabain binding to ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase EC 3.6.1.3.) were examined. Only Na^+ increased both phospho-enzyme and [^{3}H]ouabain binding livels above those observed in the presence of Mg^{2+} alone. While Na^+ stimulated phosphorylation with an apparent K_m of about 1 mM, its stimulation of [^{3}H]ouabain binding was biphasic, the lower K_m for stimulation corresponding to the K_m for formation of phospho-enzyme. Among the other alkali metal cations, potassium, rubidium and lithium were at least eight times more effective in reducing phospho-enzyme levels than in reducing [^{3}H]ouabain binding. This discrepancy is not due to the stability of the enzyme-oubain complex, nor to any action on the rates of formation or dissociation of the enzyme-ouabain complex. The data thus suggest that [^{3}H]ouabain interacts with the K^+, Rb^+ or Li^+-enzyme complexes. For Li^+, this hypothesis is further supported by the observation that Li^+ can directly increase the equilibrium level of [^{3}H]-ouabain binding to this enzyme under certain conditions.

Introduction

Studying the interactions of monovalent cations and ouabain with ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3), Sen et al. [1] showed that, in the presence of ATP, the effects of Na^+ and K^+ on the interaction of ouabain with this enzyme qualitatively paralleled their actions on the phosphorylated intermediate of this enzyme. Further studies by Barnett [2] brought forth a

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quantitative model for the interaction of ouabain with the phosphoform of $(Na^+ + K^+)$-ATPase. Barnett suggested that K^+ antagonized [^{3}H]ouabain binding to this enzyme by reducing the amount of phospho-enzyme available for ouabain interaction [2]. None of these investigators, however, directly compared the effects of alkali metal cations on phospho-enzyme levels and [^{3}H]ouabain binding.

More recently, Post et al. [3] have shown that the rate-limiting step in the reaction cycle of $(Na^+ + K^+)$-ATPase is the dissociation of potassium from the $K \cdot E_2$ enzyme complex. Thus, it is probable that during the normal turnover cycle of this enzyme a considerable proportion of the enzyme exists in the $K \cdot E_2$ form, whose reactivity with cardiac glycosides has not been determined. Li^+ can dephosphorylate $(Na^+ + K^+)$-ATPase but has minimal ability to form a stable $Li \cdot E_2$ complex, whereas Rb^+ forms a stable $Rb \cdot E_2$ complex [3,4]. Thus, a comparison of the actions of Li, K^+ and Rb^+ on steady state levels of phospho-enzyme and [^{3}H]ouabain binding to this enzyme might reveal the reactivity of the $K \cdot E_2$ or $Rb \cdot E_2$ enzyme-ion complexes with cardiac glycosides. The results obtained show that alkali metal cations which reduced the steady state levels of phospho-enzyme produced this effect at much lower concentrations than those required to reduce [^{3}H]ouabain binding. These results suggest that the monovalent cation-enzyme complex per se effectively binds cardiac glycosides.

Materials and Methods

Enzyme preparation and assay

Rat brain or guinea pig kidney $(Na^+ + K^+)$-ATPase was prepared as described by Akera and Brody [5]. $(Na^+ + K^+)$-ATPase activity was assayed as previously described [6] and the protein content of the enzymes was estimated by the method of Lowry et al. [7]. Total ATPase activity was between 150 and 300 μmol P_i/mg protein/h for the rat brain enzymes and between 90 and 180 μmol P_i/mg protein/h for the guinea pig kidney enzymes. For both tissues, about 90—95% of the total activity was ouabain sensitive.

Phosphorylation and [^{3}H]ouabain binding

Phosphorylation and [^{3}H]ouabain binding were carried out as described previously [6] with the exception of the experiment of Fig. 8. In this experiment, the phosphorylation reaction was stopped by rapid injection of 10 ml of ice cold 5% trichloroacetic acid solution. The reaction tubes were then centrifuged at 40 000 × *g* for 20 min to sediment the microsomal protein. The supernatant was discarded, and the sediment was resuspended in 10 ml of ice-cold 5% trichloroacetic acid solution and re-centrifuged as above. The reaction tubes were drained and rinsed with a few milliliters of ice-cold trichloroacetic acid solution. The sediment was solubilized with 2 M KOH and neutralized with HCl. The final volume was 2 ml. A 1 ml aliquot of the solution was mixed with 10 ml of scintillation fluid and counted in a Beckman DPM-100 liquid scintillation counter. The other 1 ml aliquot was used to determine the protein concentration by the method of Lowry et al. [7]. In most experiments, to allow comparison of data obtained on enzymes of differing specific activities, the

highest value in each experiment was arbitrarily set at 100% and other values expressed as a percentage of this [6].

Reagents, chemicals

[γ-^{32}P]ATP was obtained from New England Nuclear, Ltd., Boston, Mass. and was diluted with carrier ATP to give about 5×10^6 cpm per mol of ATP. [^{3}H]ouabain (New England Nuclear) was diluted with carrier ouabain to give 500 Ci/mol, the high specific activity being required by the relatively low concentration of [^{3}H]ouabain used in the initial binding rate experiments. Unless otherwise indicated, the data presented are the means of at least four separate experiments on different enzyme preparations, plus or minus the standard errors of the means (sem). Where appropriate, statistical significance was calculated by the t test, the criterion for significance being $p < 0.05$.

Results

Fig. 1 shows actions of the different alkali metal cations on the steady state levels of the phosphoenzyme and initial rates of [^{3}H]ouabain binding to rat brain ($Na^+ + K^+$)-ATPase. Rat brain ($Na^+ + K^+$)-ATPase was chosen for this experiment because the ouabain-enzyme complex is relatively stable [6], which facilitated investigation of initial rates of [^{3}H]ouabain binding. The right hand panel shows that among the alkali metal cations only Na^+ produced a significant increment in phosphorylation above that observed with Mg^{2+}. K^+ and Li^+ had little effect on the steady state level of the phosphoenzyme under the conditions of Fig. 1, in contrast to the actions of cesium and rubidium which depressed the steady state levels of phosphoenzyme. The left hand panel of Fig. 1 shows the initial rates of [^{3}H]ouabain binding to this enzyme observed under conditions similar to those of the phosphoenzyme experiments. The data show that only in the presence of Na^+ was the initial rate of [^{3}H]ouabain binding stimulated above that observed in the presence of Mg^{2+}. An approximately four-fold increase in the initial rate of [^{3}H]ouabain binding was observed in the presence of 100 mM Na^+, which compared well with the approximately four-fold increase in the steady state level of phospho-enzyme observed under these conditions.

Li^+ (100 mM) had little effect on either the initial rate of [^{3}H]ouabain binding to this enzyme or the steady state level of phosphoenzyme and 100 mM Rb^+ produced in a reduction in both [^{3}H]ouabain binding and steady state phospho-enzyme levels. The results with K^+ and Cs^+, however, were more complex. K^+ reduced the initial rate of [^{3}H]ouabain binding but had no effect on the steady state levels of the phospho-enzyme. Cs^+, in contrast, reduced the steady state level of phospho-enzyme compared with that observed in the presence of magnesium alone and had little effect on the initial rate of [^{3}H]ouabain binding. The data, therefore, show a relatively clear-cut action of sodium to stimulate both the steady state level of the phospho-enzyme and the initial rate of [^{3}H]ouabain binding approximately equivalently, but suggest more complex interactions with the other alkali metal cations.

The experiments with Na^+ are consistent with the hypothesis of Barnett [3] that Na^+ stimulation of phosphorylation causes the Na^+ stimulation of [^{3}H]-

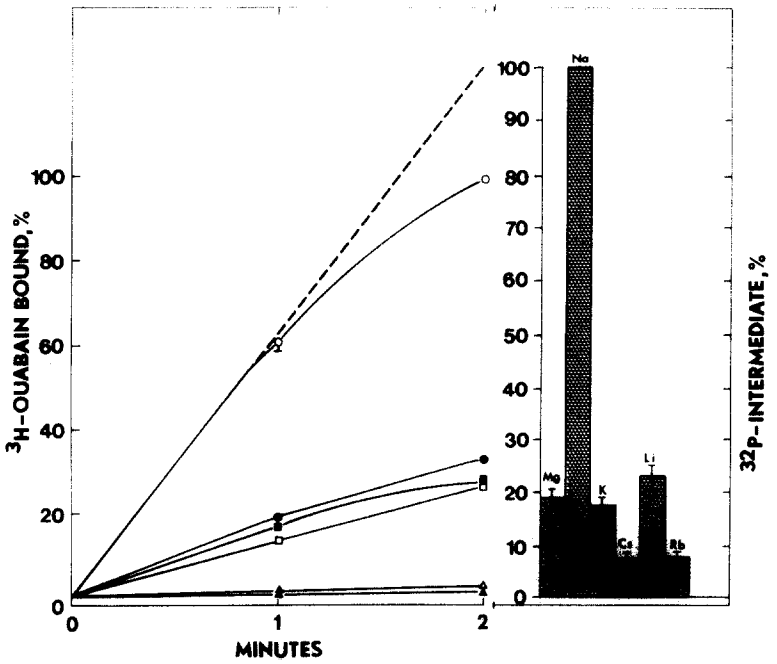

Fig. 1. Effects of monovalent cations on the steady state levels of phosphorylation and the initial rates of [^{3}H]ouabain binding to (Na^+ + K^+)-ATPase. Left hand panel: The reaction mixture contained, in 1 ml, Tris buffer (pH 7.5 at 37°C), 5 · 10^{-8} M [^{3}H]ouabain, 150 μg of rat brain enzyme preparation, and 0.1 M of NaCl (○———○), KCl (△———△), LiCl (■———■), CsCl (□———□), RbCl (▲———▲), or H_2O (●———●). The reaction was started by the addition of 2 mM $MgCl_2$ and 0.1 mM Tris/ATP, and stopped at the indicated times. All values are calculated as a percentage of binding at 2 min in the presence of NaCl, $MgCl_2$, and ATP which averaged 110 ± 6.0 pmol ouabain per mg protein. The dotted line represents the initial rate of [^{3}H]ouabain binding in the presence of Na^+, Mg^{2+} and ATP. Right hand panel: The reaction mixture contained in 1 ml, 50 mM Tris buffer (pH 7.5 at 37°C), 150 μg of rat brain enzyme preparation and 0.1 M of the indicated monovalent cations. The reaction was started with 2 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP and terminated with 5% trichloroacetic acid at 5 s. Labelling in the presence of 0.1 M Rb^+ and 1 mM ADP was deducted as background. Phosphorylation in the presence of Na^+, Mg^{2+} and [γ-^{32}P]ATP was taken as 100%, which averaged 202 ± 24 pmol ^{32}P per mg protein.

ouabain binding. If the effect of Na^+ on the initial rate of [^{3}H]ouabain binding was dependent on sodium stimulating phosphorylation of this enzyme, one would expect both of these reactions to be similarly dependent on the concentration of Na^+. Fig. 2 shows the effects of different concentrations of Na^+ on the phosphorylation reaction and the initial rate of [^{3}H]ouabain binding to this enzyme.

In each case, the steady state level of phosphoenzyme and the initial rate of [^{3}H]ouabain binding observed in the presence of magnesium and ATP with no added Na^+ was subtracted. Since [^{3}H]ouabain binding is relatively linear over short time periods in the rat brain enzyme, 1-min incubation times were employed for studies on the initial rate of [^{3}H]ouabain binding, while the steady state levels of phospho-enzyme were those observed after a 5-s incubation period at 37°C. The data of Fig. 2 show that the Na^+ stimulation of the steady state level of phosphoenzyme saturated readily, with an apparent K_m of about 1 mM. In contrast, however, the Na^+ stimulation of [^{3}H]ouabain binding of

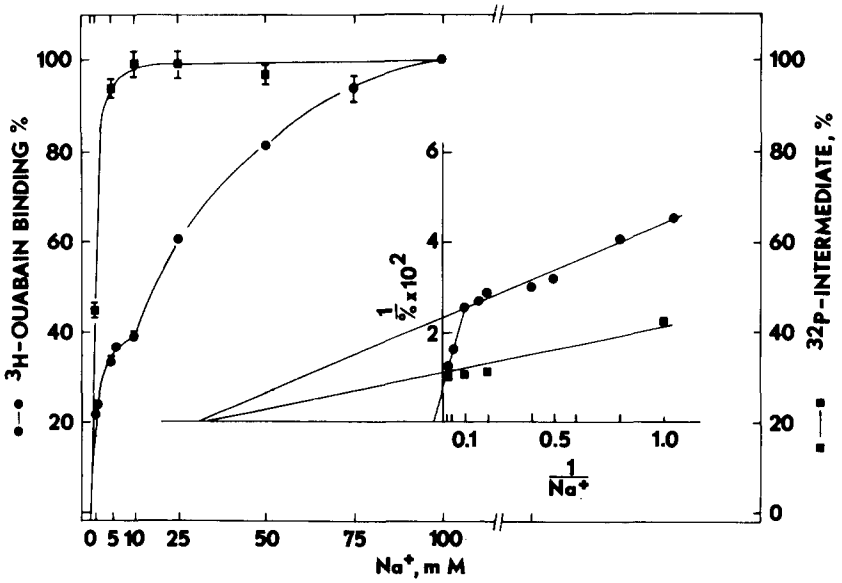

Fig. 2. Na^+ stimulation of phosphorylation and initial rate of [^{3}H]ouabain binding to (Na^+ + K^+)-ATPase. About 150 μg of rat brain enzyme were incubated with 50 mM Tris buffer (pH 7.5 at 37°C), $5 \cdot 10^{-8}$ M [^{3}H]ouabain and the indicated concentrations of NaCl. The reaction was started with 2 mM $MgCl_2$ and 0.1 mM Tris/ATP and stopped at 1 min as described in Methods. [^{3}H]ouabain binding in the presence of the indicated concentrations of Na^+ is shown by the solid circles (•———•); binding in the presence of 100 mM Na^+, 2 mM $MgCl_2$, and $5 \cdot 10^{-8}$ M [^{3}H]ouabain at 1 min is plotted as 100%, which averaged 38.53 ± 1.7 pmol ouabain per mg protein. The solid squares (■———■) show the steady state level of phospho-enzyme, formed at 5 s, under similar conditions at 37°C. The only difference was the concentration of ATP. The concentration of [γ-^{32}P]ATP used for phosphorylation studies was lower (0.05 mM) than that of Tris/ATP used for [^{3}H]ouabain-binding studies (0.1 mM). Phosphorylation in the absence of Na^+ was deducted as background, while that in the presence of Na^+ was taken as 100% which averaged 488 ± 26 pmol ^{32}P per mg protein. The insert shows a double reciprocal transformation of this data.

this enzyme was apparently biphasic with a break in the stimulation curve at about 10 mM Na^+. A double-reciprocal transformation of this data (shown in inset, Fig. 2) suggests that the effect of Na^+ on the initial rate of [^{3}H]ouabain binding can be divided into two steps, one with a K_m in the order of about 1 mM and a further step which depends on higher concentrations of Na^+. Thus, the data suggest the existence of two separate sites for the Na^+ stimulation of [^{3}H]ouabain binding to this enzyme. Since the lower K_m site corresponds well with concentrations of Na^+ which stimulate phosphorylation of this enzyme, the data are consistent with the idea that the initial step in [^{3}H]ouabain binding to this enzyme may be a Na^+ stimulation of the phosphorylation reaction. The sharp break in the double-reciprocal plot, however, indicates that there is a second mechanism by which Na^+ also affects [^{3}H]ouabain binding.

The actions of other alkaline metal cations are also complex. Fig. 3 shows the actions of different concentrations of potassium, rubidium and lithium on the steady state level of phosphorylation of this enzyme from [γ-^{32}P]ATP. Guinea pig kidney enzyme was selected for these experiments because phosphoenzyme formed with the guinea pig kidney enzyme is apparently more homogeneously E_2P phospho-enzyme than that formed with rat brain enzyme

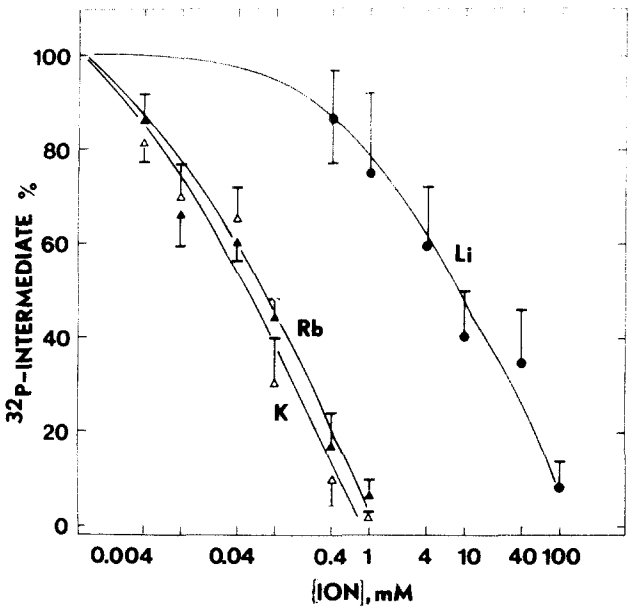

Fig. 3. Effect of K, Rb or Li on the steady state level of phosphorylation from [γ-^{32}P]ATP. The guinea pig kidney enzyme preparation was incubated at 37° with 50 mM Tris buffer (pH 7.4), 10 mM NaCl and the indicated concentrations of KCl (△———△), RbCl (▲———▲), or LiCl (●———●). The reaction was started with 2 mM $MgCl_2$ and 0.05 mM [γ-^{32}P]ATP, and stopped after 5 s. Labelling in the presence of 15 mM KCl was deducted as background. Labelling in the presence of 10 mM Na^+, 2 mM Mg^{2+}, 0.05 mM ATP was taken as 100%, which averaged 341 ± 56 pmol ^{32}P per mg of protein.

[6]. Additionally, the level of [^{3}H]ouabain binding to guinea pig kidney enzyme equilibrates more readily than that to the rat brain enzyme [8]. The data show that under these conditions potassium and rubidium reduced the steady state level of phospho-enzyme with an apparent K_i of about 0.08 mM while lithium was much less effective with an apparent K_i of about 8 mM.

Fig. 4 shows the effects of rubidium, potassium and lithium on the equilibrium levels of [^{3}H]ouabain binding to guinea pig kidney (Na^+ + K^+)-ATPase. Under conditions of this experiment, 50% inhibition of the steady state level of [^{3}H]ouabain binding to this enzyme was observed at about 0.4 mM potassium and rubidium, whereas more than 200 mM lithium was required similarly to inhibit [^{3}H]ouabain binding.

The simplest hypothesis for the mechanism of inhibition of [^{3}H]ouabain binding by potassium assumes that the phospho-enzyme is the only species which binds [^{3}H]ouabain and that potassium antagonizes binding by accelerating the breakdown of phospho-enzyme [2]. If this hypothesis were correct one might expect the concentrations of K^+ or Rb^+ which half-maximally reduce the steady state levels of phospho-enzyme to be the same as those half-maximally inhibiting [^{3}H]ouabain binding. Figs. 3 and 4, however, show that at least 5-fold greater concentrations of monovalent cations were required to reduce levels of [^{3}H]ouabain binding than the steady state levels of phospho-enzyme. Thus, these data show dissociation between the actions of these monovalent cations on phospho-enzyme and [^{3}H]ouabain binding and may suggest that

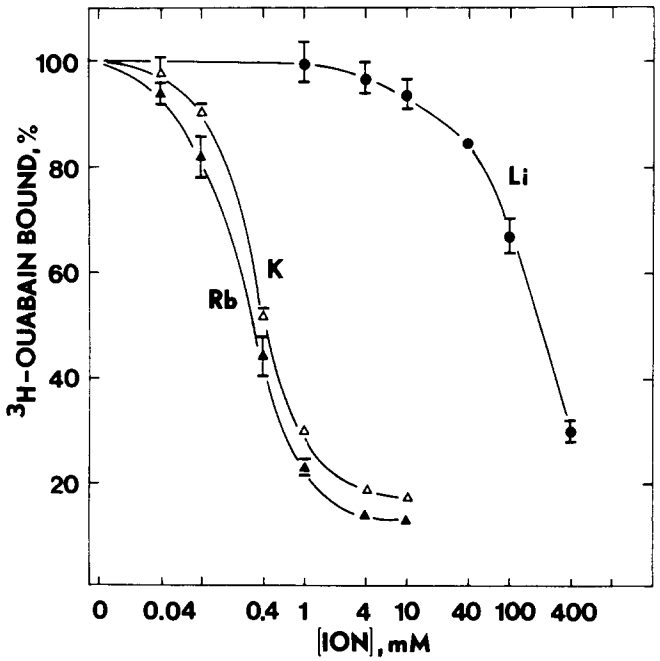

Fig. 4. Effect of K^+, Rb^+ or Li^+ on sodium-stimulated equilibrium level of [^{3}H]ouabain binding to (Na^+ + K^+)-ATPase. The guinea pig kidney enzyme preparation was incubated in 50 mM Tris buffer with 10 mM NaCl, $5 \cdot 10^{-7}$ M [^{3}H]ouabain, 3 mM $MgCl_2$, 0.2 mM Na_2ATP, together with indicated concentration of KCl (△———△), RbCl (▲———▲), or LiCl (●———●) at 37°C for 2.5 min. Binding in the absence of added K^+, Rb^+ or Li^+ was taken as 100%, which averaged 101 ± 5.0 pmol [^{3}H]ouabain per mg of protein.

[^{3}H]ouabain can interact with and bind to the various monovalent cation-enzyme complexes present in Figs. 3 and 4.

Further examination of the effects of alkali metal cations on the rates of formation and dissociation of the enzyme-ouabain complex under the conditions of Fig. 4 appeared prudent, however, before concluding that ouabain does indeed interact with the potassium, rubidium or lithium enzyme complexes. A basis for the discrepancy between Figs. 3 and 4 might be the relative stability of the enzyme-ouabain complex, which could allow a small steady state level of phospho-enzyme to support greater amounts of [^{3}H]ouabain binding [1,9]. To investigate this possibility, we studied the actions of cation concentrations which half-maximally reduced the steady state level of phospho-enzyme both on the initial rates and equilibrium levels of [^{3}H]ouabain binding. The rationale behind this approach was that if the initial rates of [^{3}H]ouabain binding were reduced considerably more than the equilibrium levels it might be possible to account for the discrepancies between Figs. 3 and 4 in terms of the relative stability of [^{3}H]ouabain binding to this enzyme. The data of Fig. 5, however, show that concentrations of Li^+ which caused more than a 50% reduction in the steady state level of phospho-enzyme had no significant effect on the initial rate of formation of the enzyme-ouabain complex and produced only minimal reductions in the equilibrium level of this binding. Similarly, concentrations of K^+ which produced a 50% reduction in the steady

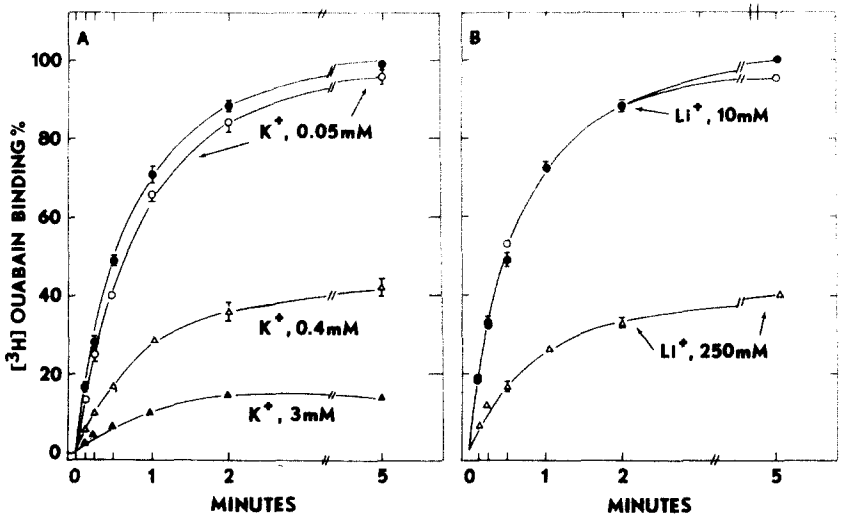

Fig. 5. Effects of K^+ and Li^+ on the formation of the enzyme-ouabain complex. Panel A. About 150 μg of guinea pig kidney enzyme were incubated with $2.5 \cdot 10^{-7}$ M [^{3}H]ouabain, 10 mM NaCl, 0.2 mM ATP and 3 mM $MgCl_2$, in the absence (●———●) or presence of 0.05 mM (○———○), 0.4 mM (△———△) or 3 mM (▲———▲) KCl. Binding is expressed as a percentage of binding at 5 min. in the absence of potassium, which averaged 137.0 ± 7.0 pmol [^{3}H]ouabain per mg protein. Panel B. All experimental conditions as for panel A except that the indicated concentrations of LiCl were substituted for potassium.

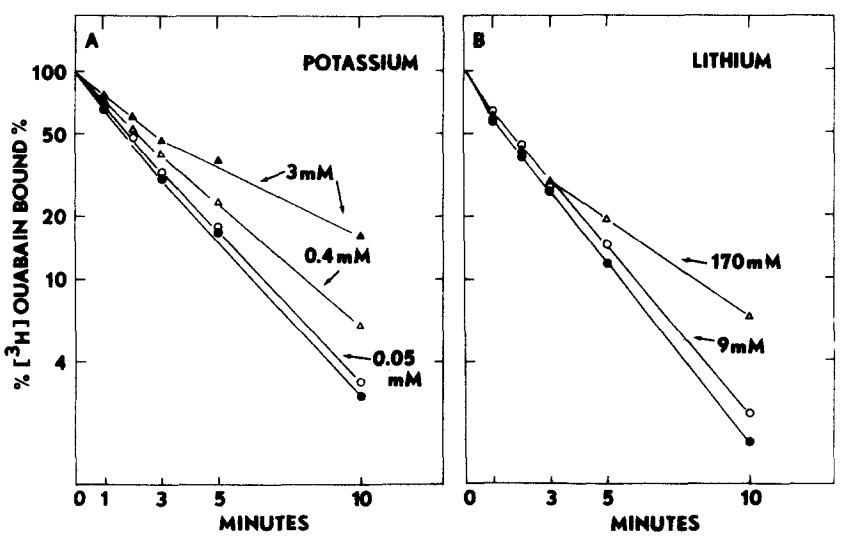

Fig. 6. Effects of K^+ and Li^+ on rates of dissociation of the enzyme-ouabain complex. Panel A. The reaction conditions were as in Fig. 5. After a 5-min pre-incubation to allow [^{3}H]ouabain binding to equilibrate, $2.5 \cdot 10^{-4}$ M unlabelled ouzbain was added to the reaction system (indicated zero time) and the reaction stopped at the indicated time points. [^{3}H]Ouabain binding was calculated as a percentage of binding at zero time. The solid circles (●———●) show the amounts of [^{3}H]ouabain remaining bound in the absence of added K^+, the open circles (○———○) binding in the presnece of 0.05 mM K^+, open triangles (△———△) binding in 0.4 mM K^+, solid triangles (▲———▲), binding in 3 mM K^+. [^{3}H]ouabain binding at zero time in the absence of added cations averaged 158 ± 6.0 pmol [^{3}H]ouabain per mg protein. Panel B. The experimental protocol was as in Panel A except that lithium was substituted for potassium. Solid circles (●———●) no added Li^+, open circles (○———○) 9 mM Li^+, open triangles (△———△) 170 mM Li^+. Binding is plotted as a percentage of that observed at zero time in the absence of added cations, which averaged 143.0 ± 7.0 pmol [^{3}H]ouabain per mg protein.

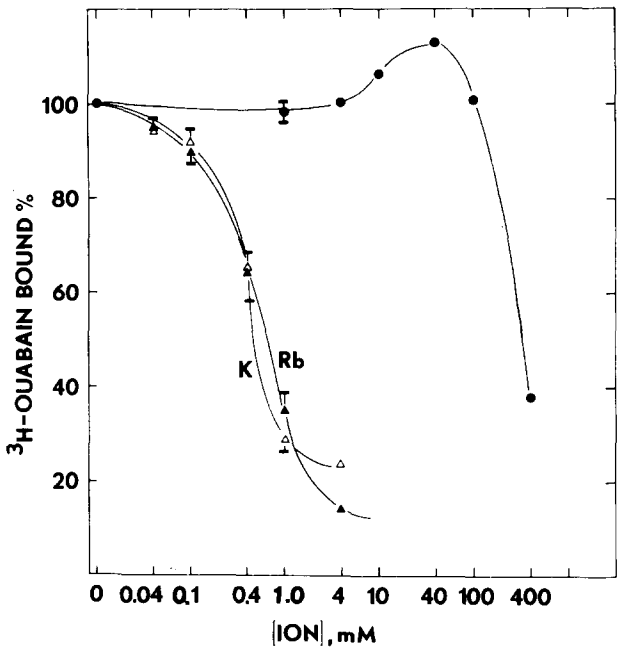

Fig. 7. Effect of Li^+ on Na^+, Mg^{2+} and ATP-stimulated [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. About 150 μg of the guinea pig ($Na^+ + K^+$)-ATPase preparation were incubated with 10 mM Na^+, 3 mM MgATP and $2.5 \cdot 10^{-7}$ M [^{3}H]ouabain in the presence of the indicated cations. The solid triangles (▲———▲) show binding in the presence of the indicated concentration of Rb^+, the open triangles (△———△) binding in the presence of K^+, the solid circles (●———●) binding in the presence of Li^+. All data points are the means of experiments on three different enzyme preparations and [^{3}H]ouabain binding is plotted as a percentage of binding in the absence of added cations, which averaged 101 ± 8.0 pmol [^{3}H]ouabain per mg protein.

state level of phospho-enzyme did not produce changes of equivalent magnitude in either the initial rate or steady state levels of ouabain binding to this enzyme. These experiments thus show that substantial reductions of the steady state levels of phospho-enzyme by potassium or lithium were observed without equivalent reductions in the initial rates or equilibrium levels of [^{3}H]ouabain binding to this enzyme.

Another explanation for the discrepancy between the cation sensitivities of phospho-enzyme and [^{3}H]ouabain binding to this enzyme would be actions of these cations to reduce the rate of dissociation of the ouabain-enzyme complex [10]. Fig. 6 shows an experiment designed to examine the actions of potassium and lithium on the rate of dissociation of the ouabain-enzyme complex under the conditions of the experiments of Figs. 3 and 4. Under these conditions, the concentrations of K^+ and Li^+ producing 50% reductions in the equilibrium levels of [^{3}H]ouabain binding (0.04 and 170 mM, respectively) produced a slight decrease in the rates of dissociation of [^{3}H]ouabain from this enzyme. Such a small magnitude of stabilization, however, is insufficient to account for the more than 8-fold difference between concentrations of cations required to inhibit the phosphorylation and [^{3}H]ouabain binding reactions of this enzyme.

The experiments of Figs. 5 and 6, therefore, seem to support the conclusion

that ouabain can interact with (Na^+ + K^+)-ATPase when it is complexed with either potassium, rubidium or lithium. Direct evidence in support of this conclusion is presented in Fig. 7. In this experiment, the actions of lithium on [^{3}H]ouabain binding in the presence of a higher concentration of ATP than that presented in Fig. 4 was investigated. Under these conditions, Li^+ produced a small but significant stimulation of [^{3}H]ouabain binding to this enzyme in the presence of sodium, magnesium and ATP. The action of Li^+ on [^{3}H]ouabain binding was again associated with an action of Li^+ to reduce the steady state levels of phospho-enzyme, as shown in Fig. 8. Since Li^+ must have interacted with the enzyme to produce these effects on both phospho-enzyme and

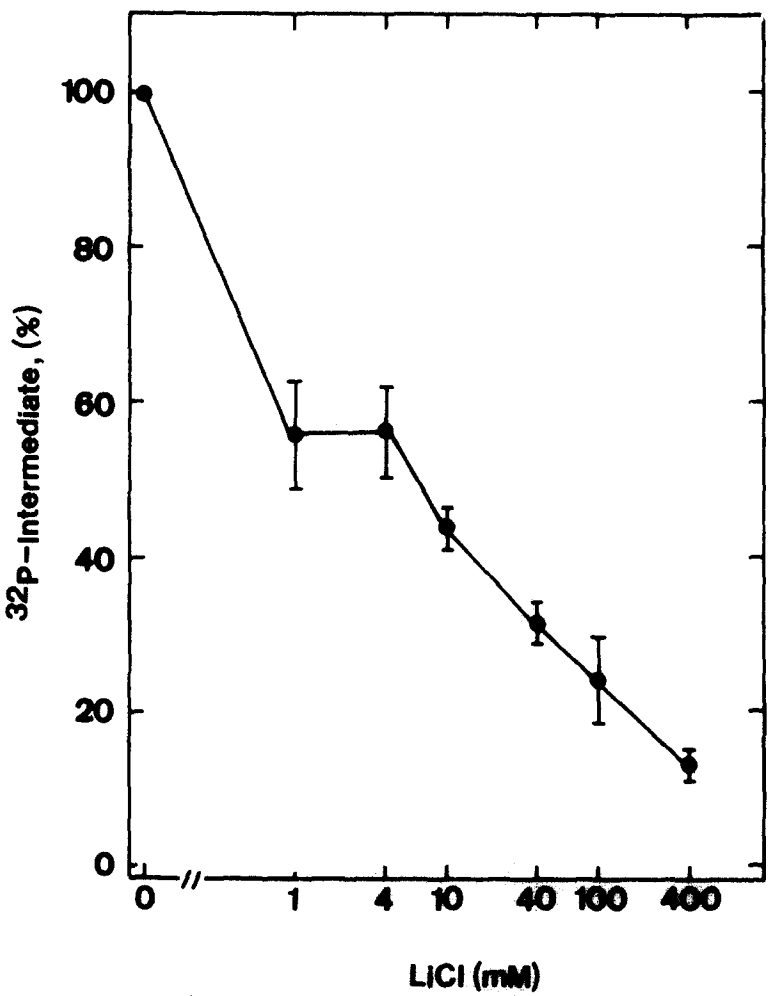

Fig. 8. Effect of Li^+ on the steady state level of phosphorylation in the presence of high concentration of [γ-^{32}P]ATP and Na^+. The guinea pig kidney enzyme preparation was incubated at 37°C with 50 mM Tris buffer (pH 7.4), 10 mM NaCl and the indicated concentrations of LiCl. The reaction was started with 3 mM $MgCl_2$ and 3 mM [γ-^{32}P]ATP and stopped after 5 s. Labelling in the presence of 2 mM ADP and 100 mM RbCl was deducted as background. Labelling in the presence of 10 mM Na^+, 3 mM Mg^{2+}, 3 mM ATP was taken as 100%, which averaged 2714 ± 62 pmol ^{32}P per mg of protein.

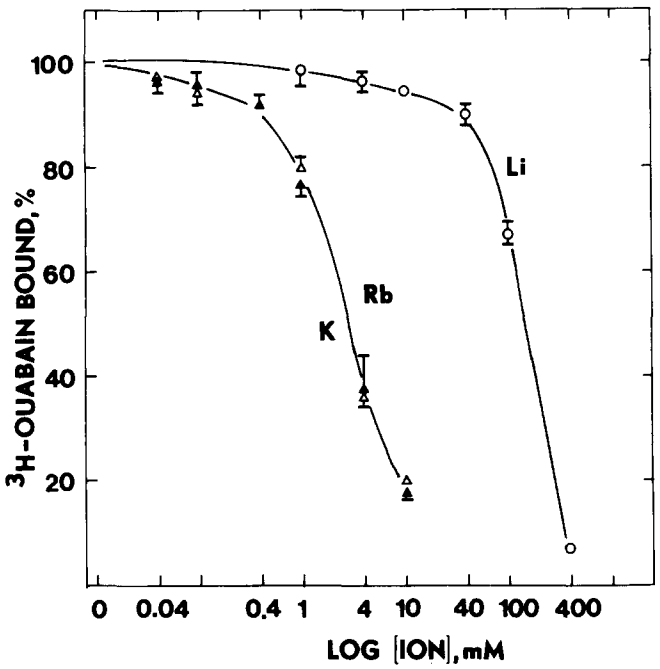

Fig. 9. Effect of K^+, Rb^+, or Li^+ on P_i-stimulated [^{3}H]ouabain binding. Guinea pig kidney enzyme was incubated with 50 mM Tris buffer, $5 \cdot 10^{-7}$ M [^{3}H]ouabain, 4 mM $MgCl_2$, 1 mM P_i and the indicated concentrations of KCl (△———△), RbCl (▲———▲), or LiCl (○———○) at 37°C for 15 min. Binding is expressed as percentage of that observed in the absence of added K^+, Rb^+, or Li^+, which averaged 151.8 ± 7.4 pmol [^{3}H]ouabain per mg protein.

[^{3}H]ouabain binding, this experiment provides further evidence for the binding of glycosides to an enzyme which has already reacted with lithium and lost its acylphosphate group.

Fig. 9 shows the actions of potassium, lithium and rubidium on the equilibrium levels of [^{3}H]ouabain binding to this enzyme in the presence of magnesium and inorganic phosphate. The data show that the Mg^{2+}- and P_i-stimulated pathway of [^{3}H]ouabain binding is more resistant to potassium and rubidium than the ATP-stimulated pathway but that these pathways are similarly sensitive to Li^+.

Discussion

Among the alkali metal cations, Na^+ is the only cation which increases the steady state levles of phospho-enzyme above those formed in the presence of Mg^{2+} [9]. Na^+ also uniquely produces this effect in enzymes pretreated with *N*-ethyl maleimide [10] and in the presence of Ca^{2+} [6]. Thus, it appears likely that its stimulation of phospho-enzyme is due to the ability of Na^+ to stimulate the formation of phospho-enzyme rather than to its relative inability to catalyze the breakdown of phospho-enzyme.

Despite the good qualitative correlation between the Na^+ stimulation of phospho-enzyme and [^{3}H]ouabain binding in Fig. 1, the ion concentration dependence of both these reactions were quite different. While the stimulation of

phosphorylation by Na^+ was approximately hyperbolic with an apparent K_m of about 1 mM, the sodium stimulation of [^{3}H]ouabain binding was more complex. Sodium in low concentrations sharply stimulated the initial rate of [^{3}H]-ouabain binding with an apparent K_m of about 1 mM and then in higher concentrations more slowly stimulated the rate of [^{3}H]ouabain binding with an apparent K_m of about 33 mM NaCl. While the lower K_m for the sodium stimulation of [^{3}H]ouabain binding corresponds well with the K_m for sodium stimulation of phosphorylation, the function of the higher K_m site seems unclear. However, because of the possibility of a metastable E_2-type enzyme or E_2-P phospho-enzyme form of the enzyme existing associated with the orientation of the sodium transport sites to the exterior, it may be that this is an effect of sodium acting at "extracellular binding sites" or, perhaps more correctly, on the enzyme in that configuration in which the sodium binding sites are oriented extracellularly. Thus, by this hypothesis the data of Fig. 2 may be accounted for in terms of sodium initially stimulating formation of the phospho-enzyme and then modulating the interaction of ouabain with the phospho-enzyme later in the transport cycle. This hypothesis may be further supported by the observations of Tobin et al. (1973) that high concentrations of Na^+ can alter the equilibrium between the E_1-P and E_2-P phospho-enzyme forms of this enzyme and the observations of Sen et al. (1969) that high concentrations of Na^+ increase the rate of stabilization of the phospho-enzyme be ouabain.

The simplest explanation for the discrepancy between the steady state levels of phospho-enzyme and equilibrium levels of [^{3}H]ouabain binding is that [^{3}H]ouabain can interact directly with and bind to the various monovalent cation enzyme complexes. The first clear-cut evidence in support of this hypothesis was that of Hansen and Skou (1973), who showed that K^+ was unable to inhibit completely Mg^{2+}- and P_i-dependent binding of [^{3}H]ouabain to ox brain ATPase. From these observations, Hansen and Skou concluded that [^{3}H]ouabain and K^+ bound to the (Na^+ + K^+)-ATPase simultaneously and these authors assumed a much higher dissociation constant for the ouabain-enzyme complex when the enzyme was saturated with K^+.

An alternative explanation for the discrepancy between the cation concentrations required to inhibit [^{3}H]ouabain binding and phospho-enzyme levels could be based on the differences between the ATP concentrations used in each case. Post et al. (1972) have shown that ATP and K^+ have antagonistic effects on the reaction cycle of (Na^+ + K^+)-ATPase, in which the rate of dissociation of K^+ is rate limiting and ATP accelerates the rate of dissociation of K^+ and thus phosphorylation of the enzyme. By this model the different effects of the alkali metal cations would simply be due to the greater concentration of ATP present in the ouabain binding system, making it more difficult for K^+ (or Rb^+ or Li^+) to reduce the steady state levels of phospho-enzyme.

There are a number of problems with this alternative hypothesis. While one can be reasonably sure of the concentrations of ATP present in the phosphorylation system, this is not the case in the [^{3}H]ouabain-binding system. This is because of the higher concentrations of enzyme present in the ouabain-binding system and the much slower time course of the [^{3}H]ouabain-binding reaction. Thus, the concentration of ATP in the [^{3}H]ouabain-binding system tends to decline rapidly and at 3—5 min after the start of the binding reaction

may be much closer to that present in the phosphorylation system, making any hypothesis dependent on the differences in ATP concentrations less attractive.

The other principal problem with the ATP concentration hypothesis is the effects of Li^+. Post et al. (1972) and Tobin et al. (1974) have shown that Li^+ has essentially no ability to antagonize the actions of ATP on either rat brain or guinea pig kidney ($Na^+ + K^+$)-ATPase. There is, nevertheless, a greater than 20-fold discrepancy between the concentrations of lithium which are required to reduce the steady state level of phospho-enzyme used in these experiments. Similarly, at higher (3 mM) concentrations of ATP, Li^+ was again about 30 times more effective in reducing the steady state level of phospho-enzyme than in reducing the equilibrium level of [^{3}H]ouabain binding. Further, in the presence of high concentrations of ATP, Li^+ actually stimulated the equilibrium level of [^{3}H]ouabain binding to this enzyme at concentrations which more than 50% reduced the steady state levels of phospho-enzyme. These results strongly suggest that the discrepancy between the concentrations of the monovalent cations which inhibit the phosphorylation and [^{3}H]ouabain binding reactions are not due to differences in the concentrations of ATP in the system and support the idea that [^{3}H]ouabain is able to interact directly with the alkali metal cation-enzyme complex.

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