REGULATORY EFFECTS OF POTASSIUM ON ($Na^+ + K^+$)-ACTIVATED ADENOSINETRIPHOSPHATASE*

G. J. Siegel, B. B. Goodwin, and M. J. Hurley

Neurology Department
University of Michigan Medical Center
Ann Arbor, Michigan 48104

Introduction

The purpose of this paper is to describe evidence that potassium ion can inhibit an early step in the reaction of nucleotide with ($Na^+ + K^+$)-activated ATPase; in addition it possesses the well known effect of activating enzyme dephosphorylation. There are two lines of evidence that bear on this issue. One comprises the observations of K^+ inhibition of hydrolysis under certain conditions and the other derives from effects of K^+ on ouabain binding to the enzyme.

For the purposes of discussion, the magnesium-dependent, ($Na^+ + K$)-stimulated ATP hydrolysis is written for each active enzyme subunit as follows. The support for this enzyme reaction model has been discussed previously.[1,2]

$$E_1 + ATP \xleftrightarrow{Mg^{2+},\ Na^+} E_1 \sim P + ADP \qquad \text{(I)}$$

$$E_1 \sim P \xrightarrow{Mg^{2+}} E_2\text{—}P \qquad \text{(II)}$$

$$E_2\text{—}P + K^+ \longleftrightarrow K\text{—}E_2\text{—}P \qquad \text{(III)}$$

$$K\text{—}E_2\text{—}P + H_2O \longrightarrow K\text{—}E_2 + Pi \qquad \text{(IV)}$$

$$K\text{—}E_2 \longleftrightarrow E_2 + K^+ \qquad \text{(V)}$$

$$E_2 \longleftrightarrow E_1 \qquad \text{(VI)}$$

This model, in fact, predicts that K^+ could inhibit reaction I by reducing the proportion of the enzyme available in the form E_1, which enters the initial reaction with nucleotide, Mg^{2+}, and Na^+. This means that the enzyme unit with bound K^+ is precluded from rephosphorylation until the K^+ dissociates. Binding of Na^+, on the other hand, permits rephosphorylation. Therefore, in this view, the completely active enzyme subunit binds Na^+ and K^+ in sequence rather than simultaneously.

There is evidence that K^+ activates the dephosphorylation of the low energy phosphoenzyme (E_2—P) and not of $E_1 \sim P$.[3,4] If it is assumed that cation translocation (or, in other terminology, reorientation of binding sites) corresponds to the principal energy-yielding step, reaction II, then K^+ is bound after Na^+ is transferred. Therefore, an implication of this enzyme reaction model for physical models of cation transport is that, for each active subunit of the enzyme complex, there is sequential transfer of Na^+ and K^+.

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Methods

Microsomes enriched in (Na^+ + K^+)-ATPase were extracted from electroplax of *Electrophorus electricus* as described.[5] Tris salts of adenosine 5′-triphosphate (ATP), cytidine 5′-triphosphate (CTP), uridine 5′-triphosphate (UTP), and adenosine 5′-diphosphate (ADP) were products of Sigma Chemical Co. In experiments comparing hydrolysis of UTP and ATP, the ^{32}Pi released from the gamma-labeled substrate was measured.[2] In experiments comparing CTP and ATP hydrolysis, released Pi was measured by a colorimetric method.[6] The usual incubation media contained 2 to 4 μg of protein, 50 to 75 mM Tris HCl (pH 7.4), 0.1 mM 2,3-dimercaptopropanol (BAL) and substrates and ions as indicated. Incubations were at 26°C for 20 or 30 minutes. In the experiments with CTP, the BAL was replaced by 0.1 mM ethylenediaminetetraacetic acid. The methods for measuring steady-state levels of ^{32}P-enzyme and for electrophoresis of labeled peptides have been described.[2]

Initial rates of [^3H]ouabain binding to microsomes were measured using the Millipore filtration method as described.[7]

K^+ Inhibition of Nucleotide Hydrolysis

K^+ inhibition of ATP hydrolysis has been observed under the condition of very low ATP concentrations.[8,9] Although these observations are compatible with the above model, the possibility is not excluded that such enzyme preparations contain two different enzymes distinguished by their opposite responses to K^+ and their affinities for ATP.

The effect of K^+ on hydrolysis rate was studied under conditions that make reaction I rate-limiting. Under this condition, there should be no effect of K^+ on the turnover rate provided that the only action of K^+ is to activate phosphoenzyme hydrolysis. Reaction I could be made rate-limiting by using low concentrations of ATP, Na^+, and Mg^{2+} or by using nucleotides for which the enzyme has very low affinity compared to ATP. In the latter case, one must demonstrate identity of the active sites.

Na^+-stimulated UTP hydrolysis was studied using the radioisotope procedure for enzyme assays. FIGURE 1 demonstrates that the electroplax microsomes catalyze Mg^{2+}-dependent hydrolysis of UTP and that Na^+ stimulates the activity.

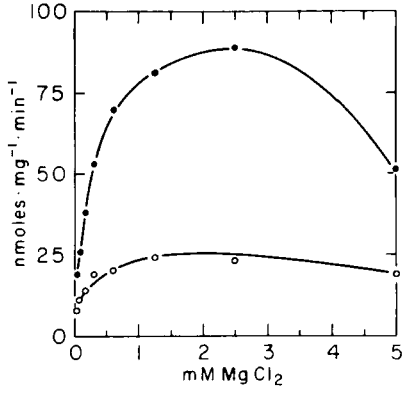

FIGURE 1. (Mg^{2+} + Na^+)-dependent UTP hydrolysis. Incubations were performed in 2.5 mM UTP for 30 minutes at 26°C. (•) 25 mM NaCl; (○) no NaCl. (From Siegel and Goodwin.[2] By permission of the publisher of *Journal of Biological Chemistry*.)

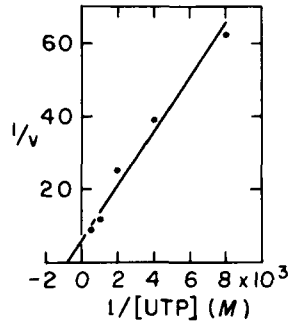

FIGURE 2. K_m for UTP as measured by Na^+-activated hydrolysis at 26°C. [$MgCl_2$] equals [UTP] plus 0.5 mM. Assays were performed with and without 25 mM NaCl and the hydrolysis rates in the absence of NaCl were subtracted from the total activity. (From Siegel and Goodwin.[2] By permission of the publisher of *Journal of Biological Chemistry*.)

The K_m for UTP is 10^{-3} as measured by Na^+-stimulated hydrolysis (FIGURE 2), and is about 1,000 times greater than reported values for ATP in the absence of K^+. The Na^+-stimulated UTP hydrolysis is completely inhibited by ouabain (TABLE 1).

FIGURE 3 compares the effect of K^+ on Na^+-activated hydrolysis of UTP and of ATP. The $(Na^+)_{0.5}$ values in the absence of K^+ are similar, about 1–2 mM. The maximum rate with UTP as substrate is about one-fourth of that with ATP, but this may be related to incomplete saturation by UTP. The addition of K^+, however, inhibits the hydrolysis of UTP. It appears that higher Na^+ levels tend to decrease the K^+-inhibition. It was also found that higher concentrations of Mg UTP decrease the inhibition.[2] In the case of ATP, the same amount of K^+ appears to inhibit at Na^+ concentrations below 1 mM but the curves cross over at 2 mM Na^+ and the same concentration of K^+ potentiates the effects of further Na^+-increments.

If UTP and ATP are reactive with the identical enzyme, it should be possible to demonstrate mutual inhibition. Since there is such a wide discrepancy in the K_m values, it is necessary to reduce the ATP to 10^{-7} M and to use dilute enzyme samples to prevent complete substrate utilization. Under this condition, 3 mM UTP inhibits both the Na^+-stimulated and the $(Na^+ + K^+)$-stimulated hydrolysis of ATP (TABLE 2). It was also found that 0.5 mM ATP completely inhibits the Na^+-activated UTP hydrolysis in 3 mM UTP.[2]

In a separate series of experiments, the Na^+ activation of CTP hydrolysis was compared to that of ATP as measured by the colorimetric determination of released Pi. FIGURE 4 shows that with CTP, the $(Na^+)_{0.5}$ is shifted from 2 mM to 5 mM by the addition of 6 mM K^+. It was found in initial trials that the K^+-

TABLE 1
Ouabain Inhibition of Na^+-stimulated UTP Hydrolysis

Prior Treatment	No Na^+	25 mM Na^+ (nmol · mg^{-1} · min^{-1})	ΔNa^+
Control	22.8	88.6	65.8
Ouabain	23.3	22.7	0

NOTE: Microsomes were previously incubated in 80 mM Tris-HCl (pH 7.4), 0.1 mM BAL, 4 mM $MgCl_2$, with or without 0.16 mM ouabain (reduced to 0.1 mM during assay) for 30 minutes at 0°C. UTP hydrolysis was then measured with or without 25 mM NaCl during 30-minute incubation at 26°C. (From Siegel and Goodwin.[2])

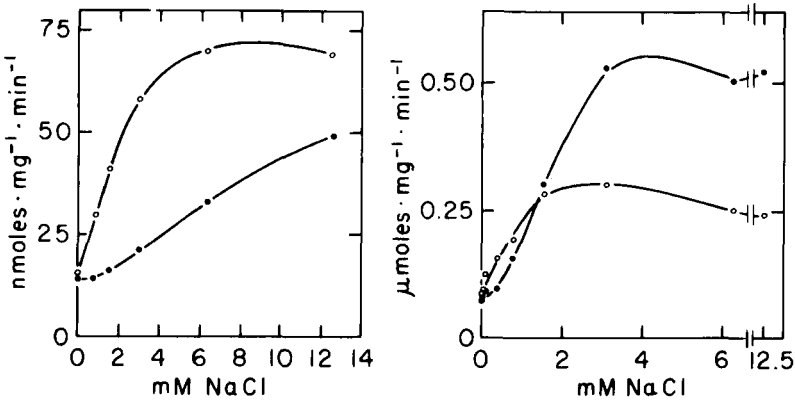

FIGURE 3. Effects of Na^+ and of K^+ on UTP and on ATP hydrolysis. Hydrolysis was measured with 30-minute incubation at 26°C. (*Left*) UTP hydrolysis; (*right*) ATP hydrolysis; (○) no KCl; (●) 6 mM KCl present. (From Siegel and Goodwin.[2] By permission of the publisher of *Journal of Biological Chemistry*.)

inhibition could be consistently demonstrated in media containing 0.5 mM CTP, 3 mM $MgCl_2$, and less than 3 mM Na^+ (FIGURE 5). The crossover point for Na^+, at which the net K^+ effect becomes stimulatory, is 3.5 mM. The Na^+ crossover point with CTP is therefore much lower than with UTP as substrate. With ATP as substrate, no K^+ inhibition is seen in this method (FIGURE 5). In some experiments employing the more sensitive radioisotope method, a crossover point may be seen at 1.5 mM Na^+ or lower (FIGURE 3).

TABLE 2
Inhibition of ATP Hydrolysis by UTP

Univalent Cations	Mg-UTP	ATP-hydrolyzed ($nmol \cdot mg^{-1} \cdot min^{-1}$) Total	Increment
A. Microsomal protein (130 ng)			
None	–	3.2 (±0.2)	
25 mM Na^+	–	15.8 (±1.3)	12.6
None	+	0.6 (±0.04)	
25 mM Na^+	+	6.6 (±0.04)	6.0
B. Microsomal protein (26 ng)			
None	–	2.4 (±0.18)	
80 mM Na^+, 20 mM K^+	–	24.4 (±1.2)	22.0
None	+	0.4 (±0.15)	
80 mM Na^+, 20 mM K^+	+	16.6 (±0.4)	16.2

NOTE: Hydrolysis of [^{32}P]ATP was measured during 10-minute incubation at 26°C in media containing 10^{-7} M [^{32}P]ATP (specific activity 3×10^6 cpm per nmol), 0.5 mM $MgCl_2$, 0.1 mM BAL, 50 mM Tris-HCl (pH 7.4), in volumes of 0.4 ml. Where indicated, 3 mM each of $MgCl_2$ plus UTP were added. NaCl and KCl were added as shown. The microsomal protein was 130 ng per sample in Part A and 26 ng in Part B. The ranges of results in duplicate samples are shown in parentheses. (From Siegel and Goodwin.[2])

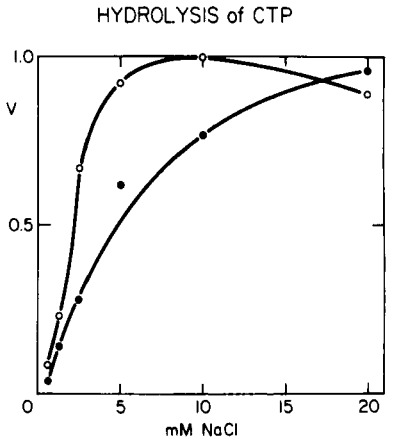

FIGURE 4. Effect of K^+ on the Na^+ activation of CTP hydrolysis. Media contained 1.25 mM CTP, 3 mM $MgCl_2$, and NaCl as shown. (○) no KCl; (●) 6 mM KCl present. Results (v) are expressed as fractions of maximal observed velocity for each condition.

The $(Na^+)_{0.5}$ values (about 1.5 mM) and the observed maximum rates in the absence of K^+ are similar for CTP and ATP (FIGURE 5). A separate confirmatory experiment shows that in 2 mM Na^+ the similarity between CTP and ATP hydrolysis rates holds at various Mg^{2+} concentrations (TABLE 3). The addition of K^+, however, inhibits the hydrolysis of CTP while stimulating that of ATP under these conditions. The K^+ inhibition is seen whether the Mg^{2+} or CTP is in excess of the other or in equal proportions (TABLE 4).

TABLE 5 shows that 1 mM CTP produces 54% inhibition of the $(Na^+ + K^+)$-activated ATP hydrolysis when the ATP is reduced to 10^{-6} M. CTP therefore competes with ATP more favorably than does UTP. This is in agreement with their respective dissociation constants.[10]

These experiments show that K^+ inhibition can be seen under appropriate conditions with UTP, CTP, or ATP as substrate. However, it appears that the relative

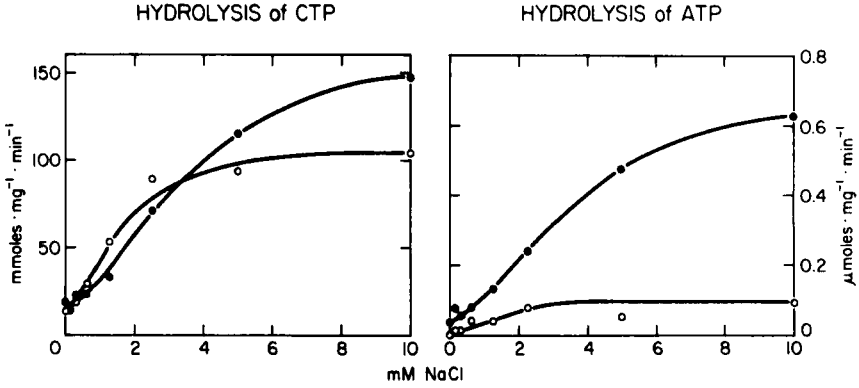

FIGURE 5. Na^+-activation of CTP and ATP hydrolysis. Media contained 0.5 mM of either CTP or ATP, 3 mM MgCl, and NaCl as shown. (*Right*) ATP; (*left*) CTP; (○) no KCl; (●) 6 mM KCl added.

TABLE 3
Inhibition of CTP Hydrolysis and Stimulation of ATP Hydrolysis by K^+

Added $MgCl_2$ (mM)	2 mM Na^+ (μmol · mg^{-1} · min^{-1})		2 mM Na^+ + 6 mM K^+ (% of control)	
	CTP	ATP	CTP	ATP
0.05	0.04	0.08	41	83
0.1	0.07	0.07	23	161
0.5	0.13	0.11	39	239
1.0	0.11	0.11	61	251
3.0	0.06	0.11	84	189

NOTE: Media contained 0.5 mM CTP or ATP, 2 mM NaCl and the indicated concentrations of $MgCl_2$. Incubations were performed for each nucleotide and Mg^{2+} concentration with and without 6 mM KCl. Activities in the presence of KCl are expressed as percentages of values obtained without KCl.

TABLE 4
K^+ Inhibition of CTP Hydrolysis in 2 mM Na^+

Additions (mM)		Hydrolysis (μmol · mg^{-1} · min^{-1})		Inhibition (%)
CTP	$MgCl_2$	Control	+6 mM K^+	
1.0	1.0	0.28	0.13	54
1.0	0.2	0.25	0.07	72
0.2	1.0	0.13	0.07	46
0.2	0.2	0.15	0.07	53

NOTE: Incubations were performed in media containing 2 mM NaCl plus the indicated concentrations of added $MgCl_2$ and CTP with and without 6 mM KCl.

TABLE 5
Effect of CTP on $(Na^+ + K^+)$-Activated ATP^{32} Hydrolysis

ATP^{32} (M)	MgCTP (M)	I*	II*	III*	Inhibition by CTP (%)
		(nmol · mg^{-1} · min^{-1})			
10^{-6}	zero	0.99	8.68	7.69	
10^{-6}	10^{-3}	0.26	3.76	3.50	54
10^{-5}	zero	3.17	34.46	31.29	
10^{-5}	10^{-3}	4.90	24.08	19.18	39

*I: 0.5 mM $MgCl_2$; II: 0.5 mM $MgCl_2$, 80 mM NaCl, 10 mM KCl; III: increment due to $(Na^+ + K^+)$.

NOTE: Microsomes, 20 ng protein, were incubated in media containing 0.5 mM $MgCl_2$ plus the indicated concentrations of [γ-^{32}P] ATP without (column I) and with (column II) the addition of 80 mM NaCl plus 10 mM KCl. 3 mM CTP plus 3 mM $MgCl_2$ were added where shown. ATP hydrolysis was measured by the release of ^{32}Pi. Inhibition by CTP is shown for the increments in hydrolysis due to Na^+ plus K^+.

potency of K^+-inhibition vs. K^+-stimulatory effects depends on the type of nucleotide, increasing in the order ATP < CTP < UTP. This order is inversely related to their respective affinities.[10]

There is evidence that [γ-^{32}P] ATP labels the phosphate acceptor site of (Na^+ + K^+)-ATPase. TABLE 6 shows that the microsomes are also labeled by [γ-^{32}P] UTP in a Na^+-dependent reaction. In comparisons of steady-state labeling it was found that at 1.4 mM nucleotide the phosphoenzyme formation with UTP is about one-half of that with ATP, that ATP blocks the formation from UTP, and that the two substrates are not additive.[2]

The Na^+-dependent phosphoprotein obtained in [γ-^{32}P] UTP media yields, upon partial proteolytic digestion with Pronase, positively charged labeled peptides with the same electrophoretic mobilities as phosphopeptides obtained similarly from microsomes phosphorylated by [γ-^{32}P] ATP in the presence of Na^+ (FIGURE 6).

TABLE 6
Phosphoprotein Formation from γ-[^{32}P] UTP

Prior Treatment	Mg-[^{32}P] UTP (mM)	No Na^+	0.15 M Na^+	ΔNa^+
		(pmol $^{32}P \cdot mg^{-1}$ protein)		
Control	0.1	15.8	64.3	48.5
	0.5	77.3	258.0	181.0
	2.0	93.0	475.0	382.0
Ouabain	0.1	40.5	37.7	0
	0.5	102.0	110.0	8.0
	2.0	167.0	200.0	33.0

NOTE: Microsomes were previously treated in 2 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), with or without 0.17 mM ouabain, for 30 minutes at 23°C. Acid-stable phosphate incorporation into protein was measured after 30-second incubation at 0°C. The concentrations of $MgCl_2$ plus [^{32}P] UTP were varied in equimolar proportions as shown. Measurements were made with and without 0.15 M NaCl. The ouabain concentration during the incubation was 0.1 mM. (From Siegel and Goodwin.[2] By permission of the publisher of *Journal of Biological Chemistry*.)

The Na^+-dependent activation of UTP hydrolysis is paralleled by increments in the steady-state level of phosphoenzyme (FIGURE 7). In addition, the K^+ inhibition of UTP hydrolysis is paralleled by inhibition of the Na^+-dependent phosphorylation (FIGURE 8). Similar relationships were found in measurements made at 0°C.[2] These studies show that Na^+ stimulation of UTP hydrolysis is proportional to increments in phosphoenzyme formation and that K^+-inhibition of hydrolysis can be related to inhibition of phosphoenzyme formation.

It was found that 0.5 mM BAL-arsenite inhibits the Na^+-stimulated UTP hydrolysis without reducing the steady-state level of Na^+-dependent phosphoenzyme, which action is identical to its action on (Na^+ + K^+)-ATPase.[4] This effect is presumed due to a block of reaction II.

From these studies with UTP, it can be concluded that the K^+-inhibitable enzyme is identical in its properties to the K^+-activatable enzyme and that only one catalytic center is involved. From this it follows that K^+ is able to inhibit an early step in the reaction of nucleotide with enzyme leading to phosphorylation.

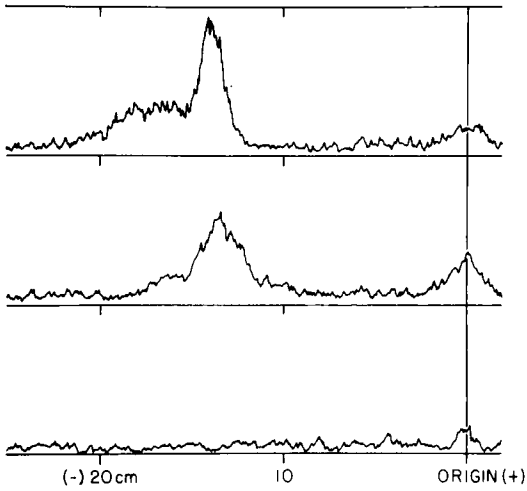

FIGURE 6. Electrophoresis of pronase-solubilized peptides labeled by ^{32}P from [γ-^{32}P] UTP and [γ-^{32}P] ATP. Microsomes, 1.9 mg of protein, were incubated in separate experiments with either 1 mM ATP or UTP in 50 mM Tris-HCl (pH 7.4) and 1 mM $MgCl_2$ with or without NaCl for 2 minutes at 0°C. The phosphorylated microsomes were treated with 1% Pronase for 30 minutes at 23°C and the solubilized peptides were subjected to electrophoresis under identical conditions. (*Top*) ATP plus 0.15 M NaCl; (*middle*) UTP plus 0.15 M NaCl; (*bottom*) UTP without NaCl. (From Siegel and Goodwin.[2] By permission of the publisher of *Journal of Biological Chemistry*.)

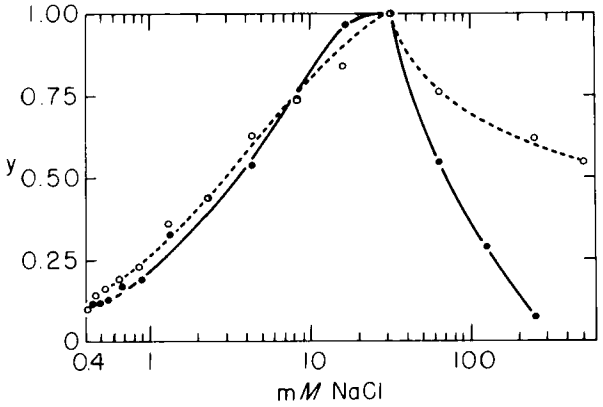

FIGURE 7. Comparison of Na^+ effects on UTP hydrolysis and on phosphoprotein levels at 23°C. (•) Hydrolysis measured 30-minute incubation at 23°C; (○) phosphoprotein levels measured at 30-second incubation at 23°C. The results (y) are expressed as fractions of the maximal velocity or phosphoprotein level observed. (From Siegel and Goodwin.[2] By permission of the publisher of *Journal of Biological Chemistry*.)

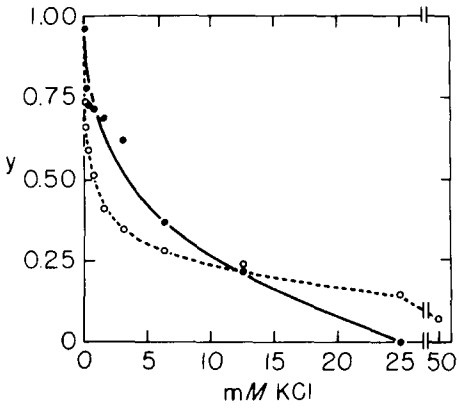

FIGURE 8. Comparison of K^+ effects on UTP hydrolysis and on phosphoprotein levels at 23°C. Incubation media in both experiments contained 0.1 mg of microsomal protein, 25 mM NaCl, 3 mM $MgCl_2$, and 3 mM UTP. Incubations were for 30-seconds at 23°C. Hydrolytic activity remaining in the presence of 0.1 mM ouabain is subtracted from the hydrolysis rates. Phosphate incorporation in the presence of 0.1 M KCl is subtracted from the phosphoprotein values. Results are expressed as in Fig. 7. •—•, hydrolysis; o---o, phosphoprotein levels. (From Siegel and Goodwin.[2] By permission of the publisher of the *Journal of Biological Chemistry.*)

This inhibition of hydrolytic rate can be seen under conditions that make reaction I rate-limiting.

The K^+ inhibition may result from decreased binding to the enzyme of Mg-nucleotide, Mg^{2+}, and/or Na^+, all of which are reactants in I. Alternatively, K^+ might not inhibit the ligand binding but instead inhibit a subsequent transfer of phosphate to the enzyme. The second case is not consistent with the reaction model, which requires E_1 to have a reduced affinity for K^+.

Although these studies do not distinguish between these alternatives, they do show that K^+ inhibition is dependent on the type of nucleotide, probably the nucleotide affinity, and the sodium concentration. Another set of investigations concerning the kinetics of ouabain binding to electroplax ($Na^+ + K^+$)-ATPase do indicate an effect of K^+ on ligand binding to the enzyme.

Ouabain Binding to ($Na^+ + K^+$)-ATPase

It is now well known that ouabain binds to particulate preparations of this enzyme from a number of tissues.† The ouabain binding to electroplax microsomes has been correlated with enzyme inhibition and appears to be specific for the ($Na^+ + K^+$)-ATPase.[7] Ouabain binding in Lubrol-solubilized supernatant fractions from electroplax microsomes can be demonstrated by gel filtration, although only a trace of enzyme activity is retained in parallel fractions after this procedure.[11] However, when the Lubrol inactivation in the 100,000 × *g* supernatant portion is greatly reduced by the addition of lecithin, 78% of the recovered enzyme activity after Sepharose 4B gel filtration is found together

†Binding of a glycoside derivative to a specific polypeptide from purified canine kidney enzyme has now been demonstrated.[18]

with 26% of recovered protein in fractions, as expected, parallel with the bound ouabain; the bound ouabain is similarly enriched, averaging 3.3 nmol • mg^{-1} protein as compared to about 0.7 nmol • mg^{-1} in native microsomes.[12] In addition, only the enzymatically active filtration fraction could subsequently bind [^{3}H] ouabain as shown by electrophoretic migration on polyacrylamide gels of a labeled band from active but not from the later inactive fractions.[12] These data taken together with the enzyme inhibition studies[1] show that there is very little, if any, nonenzyme ouabain binding in electroplax microsomes under these conditions.

There are two pathways for ouabain binding distinguishable by the opposite effects upon them of Na^+: one is dependent on Mg^{2+} plus an organophosphate ligand and is stimulated by Na^+, and the other is dependent on Mg^{2+} plus Pi and is inhibited by Na^+. K^+ inhibits both sets of reactions.[7] We are concerned here with the Na^+-stimulated pathway and its inhibition by K^+.

Initial rates of ouabain binding to microsomes were measured and plotted as fractions of saturation vs. concentrations of various ligands. FIGURE 9 shows the dependence of the rate of ouabain binding on calculated concentrations of $MgATP^{2-}$ and free ATP^{4-}. It shows that the rate of ouabain saturation increases with concentrations of $MgATP^{2-}$. However, when Mg-nucleotide dissociation yields 10^{-4} M free ATP^{4-}, the rate of ouabain binding starts decreasing and further increments of ATP lead to complete inhibition. It was found that equimolar Mg^{2+} addition reverses the inhibition by free ATP^{4-}.[7] FIGURE 10 shows that the effects of ADP are similar in that MgADP stimulates ouabain binding while free ADP^{3-} is inhibitory.

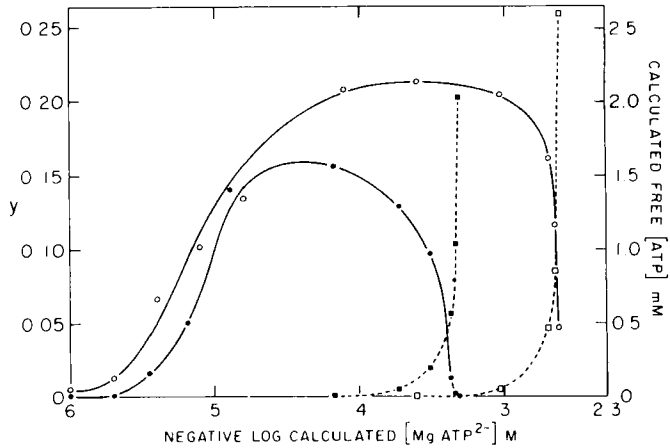

FIGURE 9. The rate of ouabain saturation as functions of calculated [$MgATP^{2-}$] and of free [ATP^{4-}]. Microsomes were exposed to [^{3}H]ouabain for 1 minute at 23°C in varying concentrations of Tris-ATP at constant levels of total $MgCl_2$. Ouabain binding is expressed as a fraction of the maximum binding capacity, y. The free [ATP^{4-}] is the difference between the calculated Mg-complex and the total ATP added. (*Solid lines*) y; (*dashed lines*) free [ATP]; (*open circles and squares*) 2.5 mM $MgCl_2$; (*closed circles and squares*) 0.5 mM $MgCl_2$. (From Siegel and Josephson.[7] By permission of the publisher of *European Journal of Biochemistry*.)

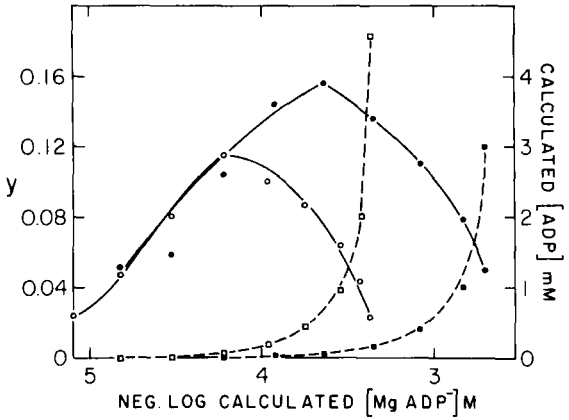

FIGURE 10. Rate of ouabain saturation as functions of calculated [MgADP] and free [ADP^{3-}]. Microsomes were exposed to [^{3}H]ouabain for 1 minute at 23°C in varying concentrations of Tris-ADP at constant levels of total $MgCl_2$. Ouabain binding is expressed as a fraction of the maximum binding capacity, y. Free [ADP^{3-}] is the difference between the Mg-complex and total ADP added. (*Solid lines*) y; (*dashed lines*) free [ADP]; (*closed circles and squares*) 2.5 mM $MgCl_2$; (*open circles and squares*) 0.5 mM $MgCl_2$. (From Siegel and Josephson.[7] By permission of the publisher of the *European Journal of Biochemistry*.)

FIGURE 11 shows that Na^+ stimulates the rate of ouabain binding in the presence of Mg^{2+} plus either ATP, ADP, or UTP. The $(Na^+)_{0.5}$ is similar in all three cases and is about 17 to 20 mM. In other experiments, Na^+ also stimulated the rate of ouabain binding in the presence of Mg^{2+} plus *p*-nitrophenylphosphate.[7] This Na^+ stimulation in the presence of Mg^{2+} plus an organophosphate most likely depends on the binding of Mg ligand to the enzyme as shown with ATP and ADP in FIGURES 9 and 10, since the free nucleotides are themselves inhibitory to ouabain binding. K^+ inhibits the rate of ouabain binding in the presence of Mg^{2+} and ATP with and without Na^+ (FIGURE 12).

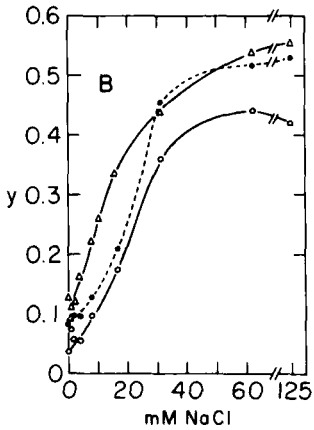

FIGURE 11. Activation of ouabain-binding rate by Na^+ in the presence of nucleotides. Microsomes were treated with [^{3}H]ouabain in 2.5 mM $MgCl_2$ and varying NaCl concentrations at constant levels of nucleotides and ouabain binding expressed as a fraction of the maximum binding capacity. Exposures in ADP and UTP were for 1 minute and in ATP for 15 seconds at 23°C. (Δ) 1.25 mM ATP; (•) 1 mM ATP; (○) 1 mM UTP. (From Siegel and Josephson.[7] By permission of the publisher of the *European Journal of Biochemistry*.)

The question we are concerned with here is whether the Na^+ stimulation of ouabain binding is due to formation of phosphoenzyme. FIGURE 11 shows that the maximum rate of ouabain binding in ATP is only about four times greater than in UTP or ADP. If phosphorylation were required for the Na^+ stimulation of ouabain binding, then one should find that ADP phosphorylates the enzyme and yields some measurable hydrolysis. Although significant ADP hydrolysis has not been observed, most media have included K^+. In analogy with K^+-inhibited UTP hydrolysis, we failed to find any ADP hydrolysis in media with and without 25 mM NaCl without added KCl[2]. Thus, any possible phosphorylation by ADP must be several orders of magnitude lower than that by ATP. UTP, on the other hand, which supports a maximum ouabain-binding rate about 20% less than does ADP, does exhibit Na^+-dependent phosphorylation and

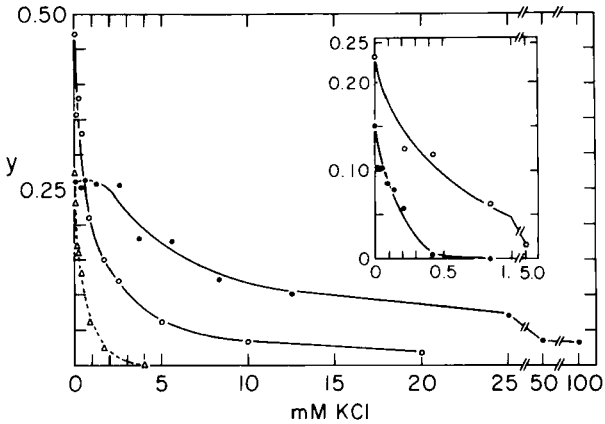

FIGURE 12. Inhibition of ouabain-binding rate by KCl. Microsomes were treated with [^{3}H]ouabain in 1.25 mM Mg ATP with and without Na^+ and varying concentrations of KCl. Ouabain binding is expressed as a fraction of the maximum binding capacity. (•) no Na^+, 23°C, 1 minute; (○) 125 mM NaCl added, 23°C, 10 seconds; (△) 125 mM NaCl, 0°C, 90 seconds. *Inset:* K^+-inhibition of ouabain-binding rate at different concentrations of NaCl. Microsomes were exposed to [^{3}H]ouabain at 0° for 90 seconds in 2.5 mM $MgCl_2$, 1.25 mM Tris-ATP and varying KCl. Ouabain binding is expressed as a fraction of maximum binding capacity. (•) 30 mM NaCl; (○) 125 mM NaCl. (From Siegel and Josephson.[7] By permission of the publisher of the *European Journal of Biochemistry*.)

Na^+-activated hydrolysis within an order of magnitude of these values for ATP. Therefore, we cannot conclude that the Na^+-stimulation of ouabain binding is dependent on enzyme phosphorylation.‡

It is more likely that ouabain binding rates measure the formation of enzyme-ligand complexes. It follows that K^+ inhibition of ouabain binding results from inhibition of the formation of the enzyme-ligand complex. A model for ouabain

‡This is still a matter of controversy. Evidence to the contrary is that ATP analogs which do not phosphorylate the enzyme also do not support ouabain binding.[19] Nevertheless, the inability to phosphorylate the enzyme is not necessarily the only functional difference between the analog and ATP, as shown by effects on Mn^{2+} binding.[20]

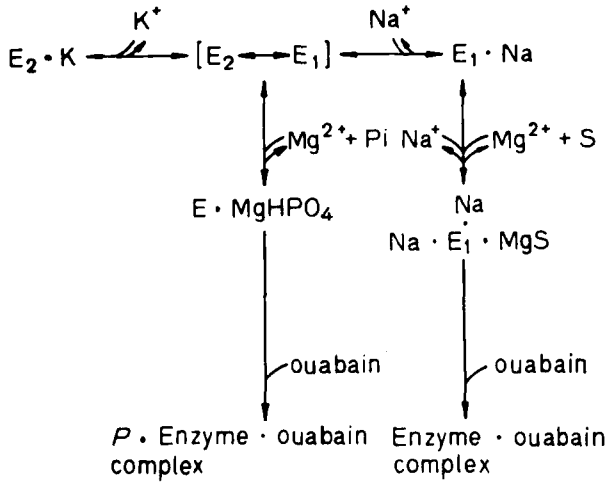

FIGURE 13. Model for ouabain binding to ($Na^+ + K^+$)-ATPase. E_1 and E_2 are conformers of the enzyme. E_1 has high affinity for Na^+ and Mg-nucleotide, but low affinity for K^+ and Pi. E_2 has high affinity for K^+ but low affinity for all other ligands. S is any organophosphate ligand that is a substrate for the enzyme. (From Siegel and Josephson.[7] By permission of the publisher of the *European Journal of Biochemistry*.)

binding based on this premise is shown in FIGURE 13. It is discussed fully elsewhere,[7] but the assumptions important for this discussion are that K^+ enzyme has decreased affinity for all ligands including Na^+ and Mg-nucleotide, while Na^+-enzyme has increased affinity for Mg-nucleotide, but decreased affinity for K^+, and also for Pi.

Discussion

The studies of K^+-inhibited nucleotide hydrolysis show that K^+ can inhibit an early reaction of nucleotide with the enzyme leading to enzyme phosphorylation. The effects of K^+ on initial rates of ouabain binding indicate that K^+ can inhibit formation of enzyme-ligand complexes involving Mg-nucleotide and Na^+. It is concluded that K^+ enzyme has decreased affinity for Na^+ and Mg-nucleotide and this accounts for the observed inhibition of enzyme phosphorylation. Direct studies of ATP binding to ($Na^+ + K^+$)-ATPase have shown that, in fact, K^+ does increase the dissociation constant for ATP[10,13] and that this effect is antagonized by Na^+.[10] The converse also has been demonstrated by kinetic studies that indicate that ATP accelerates K^+ dissociation from dephosphoenzyme.[14]

This report shows that K^+ has both inhibitory and stimulatory effects and that both are referable to the same catalytic center. The relative potencies of these two opposite effects, and hence the net effect, apparently depends on the nucleotide affinity and/or concentration. K^+ yields much greater activation in the presence of ATP than in CTP and is only inhibitory with UTP. Yet Na^+ activations in the absence of K^+ are similar with all three substrates. Thus the nucleotide-activating effect, most evident with ATP, appears independent of Na^+, but instead dependent on K^+. The nucleotide activation and K^+ inhibition

therefore appear to be aspects of the same phenomenon of K^+-nucleotide interaction.

All of these K^+-nucleotide interactions can be accounted for with the proposed equilibrium (VI) between two enzyme conformers in which E_1 has high affinity for Na^+ and Mg-nucleotide but low affinity for K^+, while E_2 has high affinity for K^+ but low affinity for Na^+ and Mg-nucleotide. In this view, K^+ binding to E_2 pulls VI to the left, decreasing the proportion of total enzyme available as E_1. This effect inhibits the Na^+-activated hydrolysis, as for example, with UTP as substrate or in low concentrations of Na^+ with CTP or in low concentrations of ATP. With sufficiently high concentrations of Na^+ and Mg-nucleotide or with nucleotide of high affinity, reaction VI is pulled to the right. This effect results in acceleration of enzyme phosphorylation (I and II) and of subsequent K^+-activated dephosphorylation in cycle (III and IV). The shift of VI to the right also accelerates the dissociation of K^+ in reaction V. The kinetic evidence for this acceleration of K^+ dissociation from dephosphoenzyme by ATP thus provides strong support for the existence of the equilibrium described in reaction VI.[14]

The negative cooperativity between K^+ and ATP results from their opposing influences on reaction VI in this model. Since, however, K^+ is an activator of IV, which in turn ensues from the ATP reaction, the shift of VI to the right can also account for activation effects by ATP as suggested.[14]

The enzymatic evidence for K^+-nucleotide interactions is pertinent to consideration of physical models for cation transport. The implications are that K^+ bound to the enzyme inhibits ATP binding and vice versa. Since Na^+ and ATP react with the same conformer and Na^+ permits enzyme phosphorylation, it follows that Na^+ and K^+ are bound sequentially with phosphorylation intervening. If reorientation of cation binding sites accompanies the principal energy-yielding step (reaction II), then it also follows that Na^+ is transferred by the subunit before K^+ is bound, i.e., that Na^+ and K^+ transfers are also sequential with respect to each active subunit.

The enzymatic reaction model is compatible with a number of physical models. However, if it is desirable to assume that there are linked, simultaneous Na^+ and K^+ transfers both directly coupled to enzyme phosphorylation, then this enzymatic model requires that at least two identical subunits, functioning cooperatively in pairs, should alternate successively between the E_1 and E_2 states. In such a model, one of the subunits (E_1) would have a high Na^+-affinity site directed inward and bound to Na^+. E_1 would be reactive with ATP. The other subunit (E_2) would have a high K^+-affinity site directed outward and bound to K^+. The tightly bound K^+ would prevent ATP binding to the substrate site on E_2. Phosphorylation of E_1 would result in reorientation of both subunits in opposite directions and reciprocal changes in the cation-site affinities. The K^+-nucleotide interactions described here would account for the reciprocating inactivation of substrate sites. This would be an example of the ligand-induced model described for enzymes exhibiting the phenomenon of "half of the sites reactivity."[15]

Application of the premise of "half of the sites reactivity" to cation transport was incorporated into physical models earlier by Stein and coworkers[16] and Repke and Schön.[17] This principle appears to be consistent with the enzymatic reaction model and the required mechanism for substrate site inactivation could be provided by the K^+-ATP negative cooperativity.

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