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Reversible Inhibition of (Na^+,K^+)ATPase by Mg^{2+}, Adenosine Triphosphate, and K^+†

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ABSTRACT: Adenosine triphosphate (ATP) hydrolysis catalyzed by the plasma membrane (Na^+,K^+)ATPase isolated from several sources was inhibited by Mg^{2+}, provided that K^+ and ATP were also present. Phosphorylation of the adenosine triphosphatase (ATPase) by ATP and by inorganic phosphate was also inhibited, as was *p*-nitrophenyl phosphatase activity. (Ethylenedinitrilo)tetraacetic acid (EDTA) and catecholamines protected from and reversed the inhibition of ATP hydrolysis by Mg^{2+}, K^+, and ATP. EDTA was protected by chelation of Mg^{2+} but catecholamines acted by some other mechanism. The specificities of various nucleotides as inhibitors (in conjunction with Mg^{2+} and K^+) and as substrates for the (Na^+,K^+)ATPase were strikingly different. ATP, ADP, β,γ-CH_2-ATP and α,β-CH_2-ADP were active as inhibitors, whereas inosine, cytidine, uridine, and guanosine triphosphates (ITP, CTP, UTP, and GTP) and adenosine monophosphate (AMP) were not. On the other hand, ATP and CTP were substrates and β,γ-NH-ATP was a competitive inhibitor of ATP hydrolysis, but not an inhibitor in conjunction with Mg^{2+} and K^+. The Ca^{2+}-ATPase from sarcoplasmic reticulum and F_1, the Mg^{2+}-ATPase from the inner mitochondrial membrane, were also inhibited by Mg^{2+}. Catecholamines reversed inhibition of the Ca^{2+}-ATPase, but not that of F_1.

It has been recognized for many years that, although Mg^{2+} is required for activity of the (Na^+,K^+)ATPase,[1] elevated concentrations of this ion inhibit the enzyme activity (Wheeler and Whittam, 1962; Dunham and Glynn, 1961; Bond and Hudgins, 1975). The mechanism and the physiological significance of this inhibition have received little attention. Mg^{2+} has also been observed to inhibit F_1, the Mg^{2+}-ATPase of the inner mitochondrial membrane (Moyle and Mitchell, 1975). There has been no attempt to compare the mechanisms by which Mg^{2+} inhibits these different ATPases. The experiments presented here address these questions and are an outgrowth of our continuing efforts to study the roles of ATPases in active transport (Knowles and Racker, 1975; Racker and Fisher, 1975; Kagawa et al., 1973; Racker, 1972), and to study the regulation of ATPases in normal and cancer cells (Scholnick et al., 1973; Suolinna et al., 1974, 1975).

We have examined the mechanism of Mg^{2+} inhibition of the (Na^+,K^+)ATPase and found that inhibition required not only Mg^{2+}, but also ATP and K^+. In comparing several different ATPases, we have found distinct differences, as well as similarities, in the mechanisms by which Mg^{2+} inhibits. The concentrations of Mg^{2+} and ATP required for inhibition of the (Na^+,K^+)ATPase were well within their physiological ranges. This admits the possibility that these ligands may be regulators of the Na^+,K^+ transport system, of which the (Na^+,K^+)ATPase is a component.

Materials and Methods

Materials

$^{32}P_i$ was obtained from ICN, Irvine, Calif. [γ-^{32}P]ATP was prepared as described by Nelson et al. (1972). Deoxycholate was obtained from Schwarz/Mann, Orangeburg, N.Y., and recrystallized as described previously (Schneider et al., 1972).

Enzyme Preparations

Lamb Kidney (Na^+,K^+)ATPase. A kidney microsomal fraction was prepared by a modification of the procedure of Grisham and Barnett (1972), and stored at −70 °C. After thawing ten kidneys for 1 h in 0.32 M sucrose, 1 mM EDTA (pH 7.0) at 4 °C, the outer medulla was dissected with scissors and homogenized in a Waring blender at 4 °C with 9 ml of 0.32 M sucrose, 1 mM EDTA (pH 7.0) per gram of tissue. The homogenate was centrifuged for 15 min at 10 500 rpm in a No. 30 Spinco rotor. The pellet was discarded and the supernatant was filtered through five layers of cheesecloth and centrifuged for 60 min at 15 000 rpm in a No. 30 Spinco rotor. The resulting pellet was resuspended in 0.32 M sucrose, 10 mM

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[1] Abbreviations used are: ATPase, adenosine triphosphatase; EDTA, (ethylenedinitrilo)tetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; P_i, inorganic phosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; ATP, CTP, UTP, and ITP, adenosine, cytidine, uridine, and inosine triphosphates; AMP and ADP, adenosine mono- and diphosphates.

Tris-Cl (pH 8.0) to 25 mg of protein per ml. While on ice, this suspension of kidney microsomes was brought to 1 M KCl by addition of solid KCl and then adjusted to 0.1 mg/ml of deoxycholate by addition of 10% sodium deoxycholate (pH 8.0). The suspension was then incubated on ice for 8 min, after which 0.2 M Na-ATP (pH 7.4) was added to bring the suspension to 3 mM Na-ATP. This suspension was then centrifuged for 60 min at 48 000 rpm in a Spinco No. 50 Ti rotor. The supernatant was carefully removed with a Pasteur pipet and discarded. The pellet was resuspended to 1–3 mg of protein/ml in 0.25 M sucrose, 10 mM Tris-Cl (pH 7.0), 3 mM Na-ATP, centrifuged as above, and resuspended and centrifuged again as described above. The final pellet was resuspended to about 25 mg of protein/ml in 0.25 M sucrose, 10 mM Tris-Cl (pH 7.0), quickly frozen, lyophilized, and stored in a desiccator at −20 °C. The ATPase activity of this final preparation was 95–99% sensitive to ouabain; the specific activity ranged from 3 to 7 μmol min^{-1} mg of $protein^{-1}$ by assay B (see below).

Other ATPase Preparations. The (Na^+,K^+)ATPase from *Electrophorous electricus* electroplax was prepared by the procedure of Albers et al. (1963), and stored at −70 °C. The Ca^{2+}-ATPase of the sarcoplasmic reticulum was isolated from rabbit back and leg muscle following procedure A of MacLennan (1970). Mitochondrial ATPase, F_1, was prepared from bovine heart by the procedure of Horstman and Racker (1970).

Assay Procedures

(Na^+,K^+)ATPase. Two different assays were used for the (Na^+,K^+)ATPase; in assay A, the release of inorganic phosphate from ATP was measured colorimetrically (Taussky and Schorr, 1953), and, in assay B, the spectrophotometric assay of Barnett (1970), ADP production was linked to NADH oxidation and monitored continuously at 340 nm. The compositions of the reaction mixtures for assays A and B were identical, except that reaction mixture B contained in addition, in a final volume of 1 ml, 20 units of pyruvate kinase, 2 units of lactate dehydrogenase, 1.5 μmol of phosphoenolpyruvate, and 0.1 μmol of NADH. The components common to both reaction mixtures were 50 mM Tris-Cl (pH 7.4), 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, and 5 mM ATP. Assay A was carried out with 20–30 μg of protein/ml at 30 °C, while assay B was carried out with about 1 μg of protein/ml at 37 °C. Specific activities were two to four times higher by assay B than by assay A, depending on the preparation and source of the enzyme.

Ca^{2+}-ATPase. Ca^{2+}-ATPase was assayed at 37 °C for 4 min (MacLennan, 1970) and phosphate release was measured colorimetrically (Taussky and Schorr, 1953). The reaction mixture contained 50 mM Tris-Cl (pH 7.5), 100 mM KCl, 50 μM $CaCl_2$, and 5 mM ATP.

Mitochondrial ATPase. F_1 was assayed for 5 min at 37 °C by the procedure of Pullman (1967), except Tris-acetate (pH 7.4) was replaced with Tris-Cl (pH 7.0). P_i released was determined colorimetrically (Taussky and Schorr, 1953).

Phosphorylation of the (Na^+,K^+)ATPase with [γ-^{32}P]ATP. (Na^+,K^+)ATPase (0.29 mg) was added to 0.9 ml of 10 mM Tris-Cl (pH 7.4), 1 mM $MgCl_2$, 16 mM NaCl with or without 2 mM KCl, and equilibrated at 0 °C for 5 min. The reaction was initiated by adding 0.4 mM [γ-^{32}P]ATP (15 cpm/pmol) and stopped 15 s later by adding 3.5 ml of ice-cold 0.25 M trichloroacetic acid, 20 mM H_3PO_4, 0.6 mM ATP (stopping solution). Aliquots of 2 ml were then filtered with suction through Gelman, Type E, 25-mm fiberglass filters. The filters were then washed with 15 ml of the stopping solution, dried, and counted. The reported values were calculated from the difference in the counts retained in the samples incubated with and without KCl.

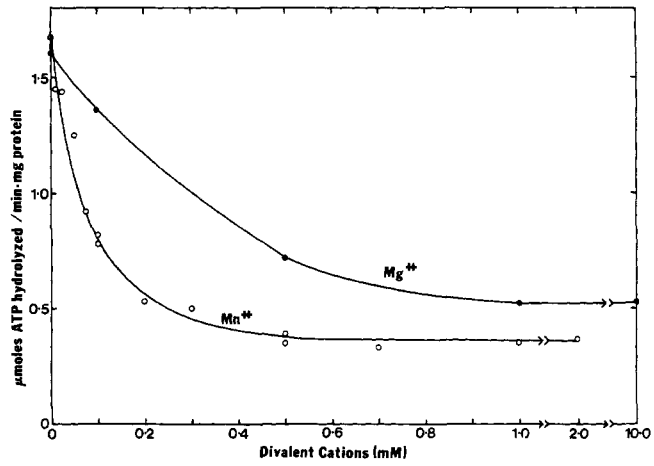

FIGURE 1: Dependency of Mg^{2+}, K^+, ATP inhibition of the (Na^+,K^+)-ATPase on Mg^{2+} and Mn^{2+} concentration. Lamb kidney (Na^+,K^+)-ATPase at 0.6 mg/ml was incubated prior to assay at 37 °C for 10 min with 5 mM Tris-Cl (pH 7.4), 10 mM KCl, 1 mM ATP, and the specified concentration of $MgCl_2$ or $MnCl_2$. Aliquots were then assayed by procedure A, except that 2 mM EDTA was also present.

p-Nitrophenyl Phosphatase. p-Nitrophenyl phosphatase activity of the (Na^+,K^+)ATPase was assayed by adding 18 μg of ATPase to a cuvette containing 1 ml of 50 mM Tris-Cl (pH 7.4), 30 mM KCl, 5 mM $MgCl_2$, and 5 mM *p*-nitrophenyl phosphate equilibrated to 30 °C. Formation of nitrophenol was monitored continuously at 400 nm with a Gilford recording spectrophotometer.

Results

Inhibition of the (Na^+,K^+)ATPase by Mg^{2+}, K^+, and ATP. When a purified preparation of (Na^+,K^+)ATPase from kidney was incubated with Mg^{2+}, K^+, and ATP prior to assay, activity was greatly inhibited. The inhibition was dependent on the presence of all three of these ligands. Figure 1 demonstrates the dependence of inhibition on the Mg^{2+} concentrations; 0.25 mM was required for half-maximal inhibition when 1 mM ATP was present. It also shows that Mn^{2+} substituted for Mg^{2+} and was effective at about fivefold lower concentrations, with 0.5 mM being required for half-maximal inhibition. Other metal ions, including Fe^{2+}, Ni^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+}, and Cu^{2+} did not substitute for Mg^{2+}, but some of these ions had other unrelated inhibitory effects on the ATPase.

The dependence of inhibition on ATP concentration is shown in Figure 2. ATP was required for inhibition but at higher concentrations it protected. The 0.5 mM concentration of ATP required for half-maximal inhibition was independent of Mg^{2+} concentration, while the concentration required for protection

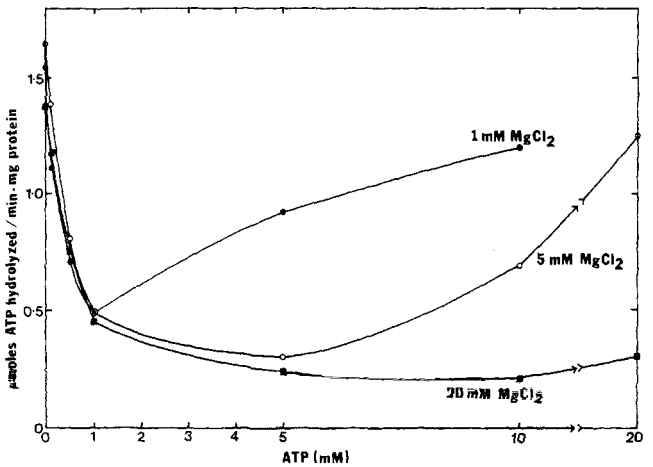

FIGURE 2: ATP dependence of Mg^{2+}, K^+, ATP inhibition of the (Na^+,K^+)ATPase. The incubation prior to assay was as in Figure 1, except the concentration of ATP was varied, while $MgCl_2$ was held constant at 1, 5, or 20 mM. Aliquots were then assayed by procedure A, except that 2 mM EDTA was also present.

TABLE I: Adenine Nucleotide Requirements for Inhibition of the (Na^+,K^+)ATPase by Mg^{2+} and K^+.[a]

Incubation Prior to Assay	ATPase (μmol min^{-1} mg of $protein^{-1}$)
5 mM Tris-Cl (pH 7.4), 20 mM $MgCl_2$	0.80
+ 5 mM ATP	0.20
+ 5 mM ADP	0.21
+ 5 mM AMP	0.81

[a] Lamb kidney (Na^+,K^+)ATPase was incubated prior to assay at 0.7 mg of protein/ml for 6 min at 30 °C with the specified components. Aliquots containing 18 μg of protein were then assayed for (Na^+,K^+)-ATPase activity by procedure A.

TABLE II: Substitution of ATP and ADP Analogues for ATP in Mg^{2+}-, K^+-, ATP-Dependent Inhibition of the (Na^+,K^+)ATPase.[a]

Incubation With or Without Nucleotide Prior to Assay	ATPase (μmol min^{-1} mg of $protein^{-1}$) Incubation With Nucleotide	Control	% Inhibition
ATP	0.19	6.45	97
β,γ-CH_2-ATP	0.20	6.43	97
β,γ-NH-ATP	3.82	2.66	<0
α,β-CH_2-ADP	2.66	7.77	66

[a] Lamb kidney (Na^+,K^+)ATPase was incubated for 6 min at 30 °C prior to assay at 0.44 mg of protein/ml with 5 mM Tris-Cl (pH 7.4), 20 mM $MgCl_2$, 10 mM KCl, and 5 mM of the specified nucleotide. Aliquots were then assayed by procedure B. The controls were samples that were not incubated with nucleotides but were assayed in the presence of the same amount of the nucleotide that was present during assay of the corresponding experimental sample.

TABLE III: Substitution of Nucleotide Triphosphates for ATP in Mg^{2+}, K^+, ATP Inhibition of the (Na^+,K^+)ATPase and the Activities of These Nucleotides as Substrates for the (Na^+,K^+)ATPase.[a]

Incubation Prior to Assay	ATPase (μmol min^{-1} mg of $protein^{-1}$)	Nucleotidase Activity with Specified Nucleotide Triphosphate as Substrate (μmol min^{-1} mg of $protein^{-1}$)
Control	4.29	
+ ATP	0.27	2.45
+ ITP	3.93	0.36
+ GTP	3.93	0.22
+ CTP	5.30	1.36
+ UTP	4.38	0.07

[a] The activity of nucleotide triphosphates as inhibitors was measured by incubating lamb kidney (Na^+,K^+)ATPase prior to assay for 10 min at 37 °C with 5 mM Tris-Cl (pH 7.4), 20 mM $MgCl_2$, 10 mM KCl, and 5 mM of the specified nucleotide. Aliquots were then assayed for ATPase activity by procedure B. The activities of nucleotide triphosphates as substrates for the (Na^+,K^+)ATPase were determined by replacing ATP in the assay mixture of procedure A with the specified nucleotide triphosphate. These assays were performed at 37 °C.

increased parallel to the Mg^{2+} concentration. This suggests that the inhibition requires free Mg^{2+} and that high concentrations of ATP prevent inhibition by chelating free Mg^{2+}.

ADP, but not AMP, substituted for ATP in inhibiting the enzyme, as is shown in Table I. Table II indicates that incubation prior to assay with β,γ-CH_2-ATP and α,β-CH_2-ADP, but not with β,γ-NH-ATP, also resulted in inhibition. Since β,γ-CH_2-ATP was not hydrolyzed by the kidney (Na^+,K^+)ATPase, but was effective in producing inhibition, it can be concluded that hydrolysis of ATP is not necessary for the inhibition. On the other hand, it is apparent that ADP inhibited directly, without conversion to ATP, because the activity of adenylate kinase in the ATPase preparation was found to be 50 times too low to generate enough ATP from ADP to cause the observed degree of inhibition. Table III shows that CTP did not substitute in inhibition for ATP even though it is a good substrate for the ATPase, being hydrolyzed at 50% of the rate of ATP hydrolysis. ITP, GTP, and UTP were neither good substrates nor inhibitors (Table III). As will be discussed later, the above data suggest that different nucleotide binding sites or specificities are involved in ATP hydrolysis and in the Mg^{2+}-, K^+-, and ATP-dependent inhibition of the ATPase.

The dependence of inhibition on the K^+ concentration is shown in Figure 3. The concentration of KCl required for inhibition was quite low; 0.1 mM gave about half-maximal inhibition, which is the same range as the K_m for K^+ for phosphoenzyme formation (Post et al., 1965) and for ATPase activity (Robinson, 1970). In order to show a requirement for K^+, the enzyme must be exhaustively washed, otherwise, sufficient K^+ is bound to the enzyme to permit inhibition.

Inhibition of the Partial Reactions of the (Na^+,K^+)ATPase by Mg^{2+}, K^+, and ATP. Table IV shows that incubating lamb kidney (Na^+,K^+)ATPase with Mg^{2+}, K^+, and ATP inhibited the known reactions and partial reactions of the enzyme. Mg^{2+}- and Na^+-dependent phosphorylation of the ATPase by [γ-^{32}P]ATP and the Mg^{2+}-dependent phosphorylation by $^{32}P_i$ were both inhibited. The K^+-dependent *p*-nitrophenylphosphatase activity was also inhibited.

Protection by Norepinephrine and EDTA from Inhibition of the (Na^+,K^+)ATPase by Mg^{2+}, K^+, and ATP. Inhibition by Mg^{2+}, K^+, and ATP was prevented by either EDTA or catecholamines, such as norepinephrine (Figure 4). However, EDTA and norepinephrine protect by different mechanisms.

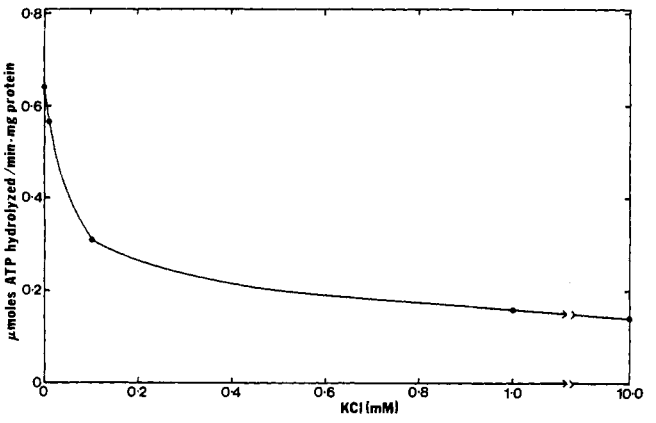

FIGURE 3: K^+ dependence of Mg^{2+}, K^+, ATP inhibition of the (Na^+,K^+)ATPase. Lamb kidney (Na^+,K^+)ATPase at 0.73 mg/ml was incubated at 30 °C for 5 min with 5 mM Tris-Cl (pH 7.4), 20 mM $MgCl_2$, 5 mM ATP, and the specified concentration of KCl. Aliquots were then assayed for ATPase activity by procedure A.

TABLE IV: Inhibition of Partial Reactions of the (Na^+,K^+)ATPase by Mg^{2+}, K^+, and ATP.[a]

Incubation Prior to Assay	pmol of P_i/mg of protein Incorp from ATP	pmol of P_i/mg of protein Incorp from P_i	(Na^+,K^+)-ATPase (μmol min^{-1} mg of $protein^{-1}$)	*p*-Nitrophenyl phosphate (μmol min^{-1} mg of $protein^{-1}$)
No incubation	491	152	3.29	0.64
10 mM K^+	471	171	2.46	
K^+ + 5 mM ATP	395	156	2.67	0.52
K^+ + 20 mM Mg^{2+}	352	232	2.35	
K^+ + Mg^{2+} ATP	0	4	0.48	0.04

[a] Lamb kidney (Na^+,K^+)ATPase at 0.45 mg/ml was incubated at 37 °C for 10 min with the specified components. This incubation mixture was then diluted eightfold with ice-cold 5 mM Tris-Cl (pH 7.4) and centrifuged 10 min at 48 000 rpm in a Spinco 50 Ti rotor and the pellet was resuspended to eightfold the original volume, centrifuged again, and resuspended to about 8 mg of protein/ml. Aliquots of this suspension were assayed for ATPase activity (procedure B) and for phosphorylation from [γ-^{32}P]ATP and from $^{32}P_i$. The *p*-nitrophenylphosphatase values are from a separate experiment in which enzyme was incubated at 0.85 mg/ml with the specified components at 37 °C for 10 min, cooled on ice, and aliquots assayed directly for *p*-nitrophenylphosphatase activity.

EDTA was required in concentrations roughly equivalent to the concentration of Mg^{2+} in excess of ATP, while norepinephrine was maximally effective at concentrations 10- to 15-fold lower than the concentration of free Mg^{2+}. EDTA probably protects by chelating Mg^{2+}, thus lowering the concentration of free Mg^{2+}, while norepinephrine must act by some mechanism other than chelation.

A survey of the effects of adrenergic activators and antagonists indicated that the effect of norepinephrine on the (Na^+,K^+)ATPase is probably not related to its physiological role. The effects of a number of adrenergic activators and related amines on Mg^{2+}-, K^+-, ATP-dependent inhibition of the (Na^+,K^+)ATPase are shown in Figure 5. Isoproterenol, a specific β agonist, and norepinephrine and dopamine, which

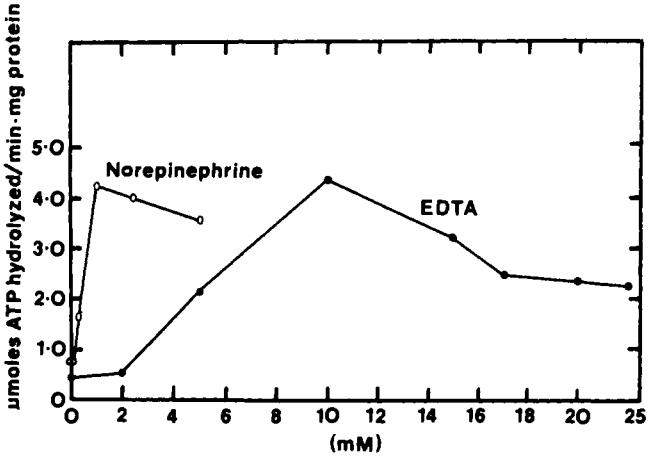

FIGURE 4: Protection by EDTA and norepinephrine from Mg^{2+}-, K^+-, ATP-dependent inhibition. Protection of the lamb kidney (Na^+,K^+)-ATPase by EDTA or norepinephrine was assessed while the ATPase assay was in progress. Assay procedure B was used, except that 20 mM $MgCl_2$ was present in order to inhibit the ATPase quickly, and the specified concentrations of EDTA or norepinephrine were added to protect from inhibition. In the absence of EDTA and norepinephrine, the reaction rate declined rapidly due to the excess of Mg^{2+}, and stabilized after 3 min to a rate of 0.4–0.7 μmol of ATP hydrolyzed min^{-1} mg of $protein^{-1}$. In the presence of EDTA or norepinephrine, the rate of reaction decreased less, resulting in rates at 3 min varying between 0.4 and 4.5 μmol, depending on the concentration of protectant present. The reaction rate in the absence of excess Mg^{2+} was about 4.5 μmol.

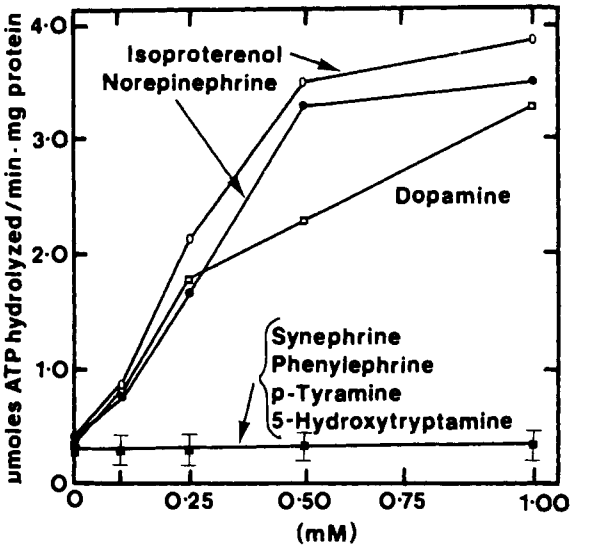

FIGURE 5: Protection of the (Na^+,K^+)ATPase from Mg^{2+}, K^+, ATP inhibition by amines. The general protocol of the experiment of Figure 4 was followed, except that the protective effects of several amines were tested, instead of EDTA and norepinephrine, and the following modification of assay procedure A was used: 7 mM ATP, 22 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 50 mM Tris-Cl (pH 7.4), 30 °C, 10-min reaction time. Phosphate was determined colorimetrically. The control activity with 7 mM $MgCl_2$ was about 4.0 μmol min^{-1} mg of $protein^{-1}$

have both α and β activity, all prevented inhibition of the (Na^+,K^+)ATPase. Epinephrine (data not shown) was also active, but many other compounds tested, synephrine, phenylephrine, tyramine, and 5-hydroxytryptamine, were inactive. It should be noted that the concentrations of catecholamines required for protection are 10 to 100 times higher than those usually required for activation of adrenergic receptors. Both α and β adrenergic antagonists partially prevented the action of norepinephrine, but the pattern of antagonist activity was

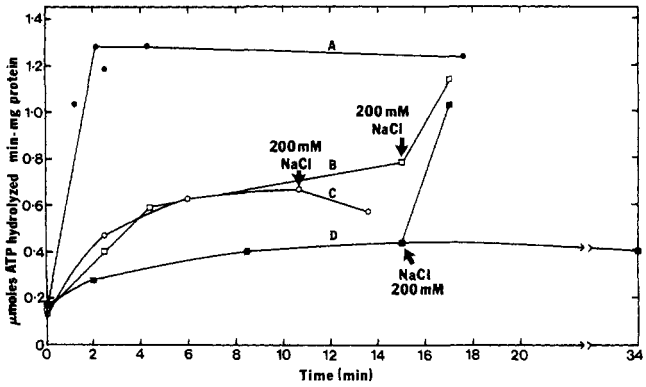

FIGURE 6: Acceleration by NaCl of the reversal of Mg^{2+}, K^+, ATP inhibition of the (Na^+,K^+)ATPase. Lamb kidney (Na^+,K^+)ATPase at 0.5 mg/ml was incubated at 37 °C for 15 min with 5 mM Tris-Cl (pH 7.4), 20 mM $MgCl_2$, 10 mM KCl, and 5 mM ATP. The enzyme was then washed free of the incubation mixture by three successive centrifugal washes with ice-cold 5 mM Tris-Cl (pH 7.4) and resuspended in the same. Aliquots of the washed inhibited enzyme were then incubated again with 5 mM Tris-Cl (pH 7.4) and 100 mM NaCl (curve A), 5 mM Tris-Cl (pH 7.4) and 10 mM NaCl (curve B), 5 mM Tris-Cl (pH 7.4) and 100 mM KCl (curve C), or 5 mM Tris-Cl (pH 7.4) alone (curve D). At the times noted by the arrows, the incubation mixtures were adjusted to 200 mM NaCl and incubation was continued. At the times specified in the figure, aliquots were assayed for ATPase activity by procedure A to assess the extent of reactivation. The specific activity of control enzyme was 1.2.

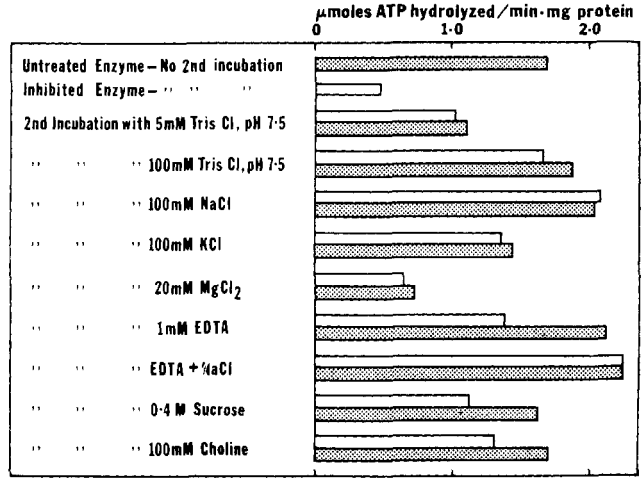

FIGURE 7: Effects of various compounds on the rate of reversal of Mg^{2+}, K^+, ATP inhibition of the (Na^+,K^+)ATPase. Lamb kidney (Na^+,K^+)-ATPase at 0.5 mg/ml was inhibited by incubation at 37 °C for 10 min with 20 mM $MgCl_2$, 10 mM KCl, 5 mM ATP, and 5 mM Tris-Cl (pH 7.5). It was then washed free of excess Mg^{2+}, K^+, and ATP, under conditions which preserved inhibition, by diluting 1:10 with cold 5 mM Tris-Cl (pH 7.5), centrifuging at 50 000 rpm in a Spinco 50 Ti rotor for 10 min and resuspending the pellet to 5.7 mg of protein/ml with ice-cold 5 mM Tris-Cl (pH 7.5). Aliquots of this inhibited enzyme were then diluted into 5 mM Tris-Cl (pH 7.5) containing the other components specified in the figure and incubated at 37 °C. After 10 (open bars) and 27 min (stippled bars) of incubation, aliquots were assayed for ATPase activity by procedure A to assess reactivation.

not characteristic of either α or β receptors (data not shown). Furthermore, as with the agonists, the concentrations required were very high.

Reversal of Mg^{2+}-, K^+-, ATP-Dependent Inhibition of the (Na^+,K^+)ATPase by Na^+ and Other Ions. The rate of reversal of Mg^{2+}-, K^+-, ATP-dependent inhibition of the kidney (Na^+,K^+)ATPase depended on the ionic composition of the medium. Figure 6 shows that dilution of inhibited ATPase into 5 mM Tris-Cl (pH 7.4) gave a small partial reversal, while 100 mM NaCl gave very rapid and complete reversal. The rate of reversal was much slower in 10 mM NaCl. KCl (100 mM) caused slow reactivation; the extent of this reactivation was less than that caused by 100 mM NaCl. NaCl (200 mM) added to the 5 mM Tris-Cl or 10 mM NaCl samples markedly increased the rate and final extent of reactivation, but did not increase the extent of reactivation in the presence of 100 mM KCl. Figure 7 surveys the effectiveness of various compounds in accelerating the rate of reactivation. Again, dilution into KCl caused partial reactivation, which was nearly complete by 10 min. Dilution into sucrose or choline-Cl caused very slow reactivation, which was incomplete in 10 min. At 27 min, the extents of reactivation with sucrose and choline-Cl were approaching the activity of the control enzyme. Dilution into 20 mM MgCl largely prevented reactivation. Dilution into 100 mM Tris-Cl, 1 mM EDTA, or 100 mM NaCl not only gave complete reactivation but significantly increased activity above the control level. The rate of reactivation with NaCl and Tris-Cl was much greater than that with EDTA. These results suggest that reactivation depends primarily on the rate of displacement of Mg^{2+} from an inhibitory site on the ATPase, since Mg^{2+} prevents reactivation and Mg^{2+} chelators accelerate it. It is also clear from these results that, although partial reversal occurs following simple dilution or following treatment with high concentrations of several solutes (sucrose, choline-Cl, KCl), maximum reactivation occurs only through the action of Mg^{2+} chelators, such as EDTA or Tris, or in the presence of Na^+.

Comparison of Mg^{2+} Inhibition of Several ATPases. Table V summarizes the effects of high concentrations of Mg^{2+} and of norepinephrine during assay of the (Na^+,K^+)ATPases from the electric organ of *Electrophorous electricus* and from lamb kidney, of the Ca^{2+}-ATPase of rabbit muscle sarcoplasmic reticulum, and of the ATPase (F_1) from bovine heart mitochondria. F_1 was inhibited by high Mg^{2+}, but norepinephrine did not prevent the inhibition. Norepinephrine partially prevented inhibition of the Ca^{2+}-ATPase and the kidney preparation of (Na^+,K^+)ATPase, while it almost completely prevented inhibition of the *E. electricus* (Na^+,K^+)ATPase.

The Mg^{2+} inhibition of F_1 has been characterized by Moyle and Mitchell (1975) and seems to differ in several respects from the Mg^{2+}-dependent inhibition of the (Na^+,K^+)ATPase and of the Ca^{2+}-ATPase. Besides the lack of protection of F_1 by norepinephrine, shown above, SO_3^{2-}, SO_4^{2-}, and HCO_3^- reversed the inhibition of F_1 by Mg^{2+}, whereas these anions did not reverse the inhibition of (Na^+,K^+)ATPase or of the Ca^{2+}-ATPase (data not shown).

The rate of reactivation following Mg^{2+}-dependent inhibition varied considerably with the tissue from which the ATPase was derived. This can be seen by comparing Tables V and VI. Table VI shows that, when the electric organ, kidney, and sarcoplasmic reticulum ATPase preparations were incubated under the inhibitory conditions described in the legend to Table V, and then diluted to reduce the Mg^{2+} concentration and assayed under noninhibitory conditions, only the lamb kidney preparation remained inhibited. Thus, inhibition was much more rapidly reversed by dilution of the electric organ and sarcoplasmic reticulum ATPases than by dilution of the lamb kidney ATPase.

Discussion

We have studied inhibition of the (Na^+,K^+)ATPase by Mg^{2+}. The results presented here establish the requirements for this inhibition and its reversal.

TABLE V: Protection from Mg^{2+}-Dependent Inhibition by Norepinephrine—Comparison of Four ATPases.[a]

Reaction Conditions	μmol of ATP hydrolyzed min^{-1} mg of $protein^{-1}$			
	Electric Organ	Kidney	Sarcoplasmic Reticulum	Mitochondria
Control	2.54	6.89	13.1	116
+ 20 mM $MgCl_2$	0.29	0.75	4.8	75
+ 20 mM $MgCl_2$, norepinephrine	2.12	4.25	8.6	75

[a] As described in the legend of Figure 4, protection by norepinephrine was assessed while the ATPase assay was in progress. The reaction conditions for each enzyme preparation were as described under Materials and Methods, except for the additions of $MgCl_2$ and norepinephrine specified below. The norepinephrine concentrations were 2 mM for F_1 and the Ca^{2+}-ATPase, 1 mM for the kidney, and 0.5 mM for the electric organ (Na^+,K^+)ATPase. Assay procedures A and B were used, respectively, for the electric organ and kidney (Na^+K^+)ATPases.

TABLE VI: Rate of Reversal of Mg^{2+}-Dependent Inhibition—Comparison of Three ATPases.[a]

Incubation Prior to Assay	μmol of ATP hydrolyzed min^{-1} mg of $protein^{-1}$		
	Electric Organ	Sarcoplasmic Reticulum	Kidney
Control	2.54	11.4	1.27
20 mM $MgCl_2$, 10 mM KCl, 5 mM ATP	2.07	9.4	0.37

[a] ATPases at 0.1–0.5 mg of protein/ml were incubated for 10 min at 37 °C with 5 mM Tris-Cl (pH 7.5) and the other specified components. Aliquots of these incubation mixtures were then diluted to decrease the Mg^{2+} concentration to relatively noninhibitory levels and assayed for ATPase activity under noninhibitory conditions. Assay A was used for (Na^+,K^+)ATPase determinations.

Our initial finding, that Mg^{2+} inhibition of the lamb kidney (Na^+,K^+)ATPase was a slowly reversible process, allowed us to use uniform assay conditions in testing the effects of different ligands on Mg^{2+} inhibition. Using this approach, we have shown that Mg^{2+} inhibition of the (Na^+,K^+)ATPase depends not only on Mg^{2+}, but also is absolutely dependent on K^+ and ATP and on no other ligands. There are two models which would be consistent with the Me^{2+} and ATP titrations shown in Figures 1 and 2. In the first formulation, free Mg^{2+} is inhibitory, and excess ATP protects from inhibition by chelation. In the second model, Mg-ATP is inhibitory and free ATP protects. Though our experiments do not conclusively distinguish between these two alternatives, two lines of evidence suggest that the first model is correct. First, the K_i values for Mg^{2+} and ATP are different, suggesting that they act separately and not as a binary complex. Second, the K_i for ATP was the same for Mg^{2+} and Mn^{2+} in spite of the fivefold difference in the K_i values for these cations. This result would be very difficult to reconcile with the second model, when one takes into account that the stability constants of Mg-ATP and Mn-ATP are almost identical.

As is suggested by the data in Table II and borne out in Lineweaver–Burk plots (data not shown), β,γ-NH-ATP is a good competitive inhibitor of the ATPase, but substitutes poorly for ATP in Mg^{2+}, K^+, ATP inhibition. In contrast, β,γ-CH_2-ATP is a poor competitive inhibitor, but substitutes quite well for ATP in Mg^{2+}, K^+, ATP inhibition. If competitive inhibition can be taken as a measure of the ability of a nucleotide to interact with the ATPase in the same way that a substrate would interact with it, then these results in conjunction with those in Table III, which survey the activities of various nucleotide triphosphates as substrates and as inhibitors, indicate that the nucleotide specificities for activity in Mg^{2+}, K^+, ATP inhibition and for substrate activity are very different. From this, we can conclude that either the nucleotide binding site for Mg^{2+}, K^+, ATP inhibition is distinct from the catalytic site of the ATPase, or there is a single nucleotide binding site which has different specificities for hydrolysis and for inhibition (in conjunction with Mg^{2+} and K^+).

The studies of reversal of Mg^{2+}-, K^+-, ATP-dependent inhibition provide further information about the inhibited state of the ATPase. Placing inhibited ATPase in medium free of Mg^{2+}, K^+, and ATP causes slow, partial reactivation and this reversal is prevented by Mg^{2+}. Thus, in the absence of the other ligands required for inhibition, Mg^{2+} is sufficient to maintain the inhibited state of the enzyme. This means either that K^+ and ATP are needed only to initiate Mg^{2+} binding to the inhibitory site, or that the inhibitory K^+ and ATP are bound extremely tightly to the ATPase and, therefore, do not dissociate significantly under the conditions tested. From our experiments, it is not clear how NaCl reverses Mg^{2+}, K^+, ATP inhibition. It is possible that Na^+ could be displacing either Mg^{2+} or K^+ from their inhibitory sites.

Independently of our observations, it has been reported (Yoshimura, 1973) that catecholamines stimulate the (Na^+,K^+)ATPase. The evidence presented here suggests, however, that catecholamines do not stimulate the (Na^+,K^+)-ATPase directly, but prevent the partial inhibition of the ATPase by Mg^{2+}, K^+, and ATP. The detailed mechanism of this protection is yet unknown, though it is clear that catecholamines do not protect by chelating Mg^{2+}, as EDTA does.

Our examination of the partial reactions of the (Na^+,K^+)-ATPase leads us to the conclusion that incubation with Mg^{2+}, K^+, and ATP converts the enzyme to a form which is unable to participate in any segment of the catalytic cycle of Na^+,K^+ dependent ATP hydrolysis.

Post et al. (1975) have observed an ADP- and K^+-insensitive phosphorylated form of the (Na^+,K^+)ATPase by treating phosphoenzyme formed by ATP in the presence of high Mg^{2+} with cyclohexanediaminetetraacetic acid, K^+, and ADP. Formation of this enzyme was blocked by Na^+ and dependent on high Mg^{2+}. Post et al. (1975) believe that this K^+- and ADP-insensitive phosphoenzyme accounts for the inhibition of the (Na^+,K^+)ATPase by excess mg^{2+}, which many investigators have reported (Wheeler and Whittam, 1962; Dunham and Glynn, 1961; Bond and Hudgins, 1975). There appears to be a striking parallel between the properties of the Mg^{2+}, K^+, ATP inhibited state of the ATPase which we have reported here and those of the K^+- and ADP-insensitive phosphoenzyme reported by Post et al. (1975).

Moyle and Mitchell (1975) propose that there are two populations of the mitochondrial ATPase existing in equilibrium with each other, one population that is active and the other that is virtually inactive in ATP hydrolysis. They further

propose that Mg^{2+} inhibits F_1 by shifting the equilibrium towards the inactive form. We suggest that a similar model can be applied to the (Na^+,K^+)ATPase, except that, in the case of this enzyme, ATP and K^+, as well as Mg^{2+}, are required to shift the equilibrium towards the inactive form. It should be pointed out, however, that F_1 contains firmly bound ATP (Rosing et al., 1975) and that all of the work by Moyle and Mitchell was done in the presence of K^+, so that the similarity of the inhibition processes for F_1 and for the (Na^+,K^+)ATPase may be greater than is apparent from the reported requirements for added factors.

Note Added in Proof

While this paper was in press, similar studies performed with dog kidney enzyme were published (Cantley, L. C., Jr., and Josephson, L. (1976), *Biochemistry 15,* 5280–5287). Drs. Cantley and Josephson (personal communication) have recently discovered that a nucleotide other than ATP, which is present in commercial ATP preparations and difficult to separate from ATP, is responsible for the inhibition of ATPase activity.

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CORRECTIONS

The Purification and Partial Characterization of a Soluble Elastin-like Protein from Copper-Deficient Porcine Aorta, by L. B. Sandberg, N. Weissman, and D. W. Smith, Volume 8, Number 8, August 1969, page 2940.

Specimen 3 of Figure 2 was prepared according to *J. Biol. Chem. 247,* 2427 (1972), rather than the method described.

Asymmetry and Transposition Rates of Phosphatidylcholine in Rat Erythrocyte Ghosts, by Bernabé Bloj and D. B. Zilversmit,* Volume 15, Number 6, March 23, 1976, pages 1277–1283.

On page 1278, lines 2, 3, and 4 at the top of column 2 should be replaced by: "30 000 × *g* for 15 min. To prepare inside-out vesicles, each milliliter of packed membranes was diluted in 40 ml of 0.5P8 and incubated for 1–3 h at 0 °C. The membranes".

Studies on the Intramolecular and Intermolecular Kinetic Isotope Effects in Pyruvate Carboxylase Catalysis, by Yak-Fa Cheung and Christopher Walsh,* Volume 15, Number 17, August 24, 1976, pages 3749–3754.

On page 3753, the statement in line 2 should read ". . . and K_m 7.1×10^{-4} M."

Molecular Topology of the Photosynthetic Light-Harvesting Pigment Complex, Peridinin-Chlorophyll a-Protein, from Marine Dinoflagellates, by Pill-Soon Song,* Prasad Koka, Barbara B. Prézelin, and Francis T. Haxo, Volume 15, Number 20, October 5, 1976, pages 4422–4427.

In Table I, ms for fluorescence lifetimes should read ns.

Sulfhydryl Group Modification of Sarcoplasmic Reticulum Membranes, by Alexander J. Murphy, Volume 15, Number 20, October 5, 1976, pages 4492–4496.

In Table I, column 2, the values in parentheses should read: (>20).