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Calcium Ion and Sodium- and Potassium-Dependent Adenosine Triphosphatase: Its Mechanism of Inhibition and Identification of the E_1—P Intermediate

T. TOBIN, T. AKERA, S. I. BASKIN, AND T. M. BRODY

Department of Pharmacology, Michigan State University, East Lansing, Michigan 48823

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SUMMARY

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Calcium ion inhibits (Na^+ + K^+)-ATPase (EC 3.6.1.3) primarily by reducing the rate of the phosphorylation step. Inhibition of this step is competitive with respect to Na^+ and noncompetitive with respect to Mg^{++}. Although Ca^{++} acts to reduce the rate of the phosphorylation step, high concentrations are required to reduce the steady-state levels of phospho-enzyme, and Ca^{++} alone stimulates a slow phosphorylation of the enzyme. In the presence of Ca^{++} and Mg^{++} the phospho-enzyme formed is sensitive to K^+ and ouabain, and its steady-state levels are low when K^+ is present. If the concentration of Mg^{++} is low, the phosphoenzyme formed in the presence of Ca^{++} is insensitive to K^+ and ouabain, and reacts readily with ADP. The reactivity of the Ca^{++}-dependent phospho-enzyme with ADP is reduced by the addition of Mg^{++}; conversely, the reactivity of the Mg^{++}-dependent phospho-enzyme with ADP is increased by high concentrations of Na^+. Ca^{++} also inhibits the Mg^{++}- and P_i-dependent pathway of [^{3}H]ouabain binding. The identification of an ADP-sensitive intermediate in the reaction cycle of this enzyme is reported. Its transformation to a K^+-sensitive intermediate is influenced by Na^+, Mg^{++}, and Ca^{++} in a manner consistent with proposed reaction mechanisms for this enzyme.

INTRODUCTION

The (Na^+ + K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3.) of mammalian cell membranes is generally considered to be the enzymatic basis of the "sodium pump" activity of these membranes, and its mechanism of action has been investigated in depth (1, 2). Various inhibitors of this enzyme system have proved useful in these investigations and have greatly increased our understanding of its mechanism (3–5). Calcium ion is a potent inhibitor of this enzyme system, but the mechanism of this inhibition has not been investigated in detail (6–8). In this communication we report on the mechanism of the inhibitory (6) and stimulatory (8) actions of Ca^{++} on (Na^+ + K^+)-ATPase.

Calcium ion was selected from among a number of inhibitors because it might prove useful in distinguishing among the various

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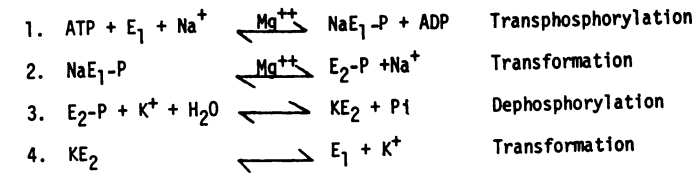

FIG. 1. *Tentative reaction cycle for $(Na^+ + K^+)$-ATPase*

The native enzyme (E_1) binds ATP and Na^+. In the presence of Mg^{++} the terminal phosphate of ATP transfers to the enzyme, forming NaE_1—PADP. ADP then dissociates from the enzyme, which in the presence of Mg^{++} stabilizes as the E_2—P form. During this transformation the affinity of the enzyme for Na^+ is reduced. The E_2—P form of the enzyme binds K^+, which accelerates the reaction of the phospho-enzyme with water (dephosphorylation). After the release of P_i the enzyme restabilizes as the E_1 form, which accelerates the dissociation of K^+ and completes the reaction cycle.

phospho forms (E—P)[1] of $(Na^+ + K^+)$-ATPase (3, 9, 10). The E_1—P form of this enzyme is considered to be a "high-energy" form existing transiently after transphosphorylation of the enzyme by Na^+, Mg^{++}, and ATP. It reacts readily with ADP and less readily with K^+ (3). In the presence of Mg^{++} E_1—P apparently undergoes a configurational change and becomes reactive with K^+ and resistant to ADP (3, 9). The latter form, designated E_2—P, is the one usually observed in the presence of Na^+, Mg^{++}, and ATP and the absence of K^+ (3). However, direct identification of the transient E_1—P form has been difficult, and to date the principal evidence for its existence has been either indirect (3, 9) or obtained in enzymes irreversibly inhibited with *N*-ethylmaleimide (3). These relationships are summarized in the reaction sequence presented in Fig. 1.

One approach to the problem of isolating E_1—P would be to identify a divalent cation which would selectively inhibit the Mg^{++}-requiring E_1—P to E_2—P transformation (reaction 2); a converse method would be to obtain a divalent cation that could substitute for Mg^{++} in the transphosphorylation reaction (reaction 1) without catalyzing reaction 2. Calcium ion was selected because it is a potent inhibitor of the over-all $(Na^+ + K^+)$-ATPase reaction (6) but does not inhibit the phosphorylation of this enzyme (11). In this communication we present evidence that Ca^{++} may stimulate reaction 1 and inhibit reaction 2 under appropriate conditions.

The rat brain enzyme is particularly suitable for investigations on the E_1—P $\rightleftharpoons$ E_2—P transformation. Whereas in guinea pig kidney $(Na^+ + K^+)$-ATPase less than 10% of the phospho-enzyme observed under standard phosphorylation conditions appears to be in the E_1—P form,[2] it appears that in rat brain enzyme, in the presence of 120 mM Na^+, a considerable proportion of the phospho-enzyme is ADP-sensitive. The proportion of the enzyme in the E_1—P form can be further varied by altering the Mg^{++}, Ca^{++}, and Na^+ concentrations in a manner consistent with the proposed roles for Mg^{++} and Na^+ in the reaction mechanism of this enzyme. These experiments also provide evidence that the E_1—P to E_2—P transformation is associated with a change in the affinity of the enzyme for Na^+, consistent with suggestions that this transformation is involved in Na^+ transport. Finally, in the presence of Na^+, Mg^{++}, and ATP, ouabain has been suggested to interact primarily with the E_2—P form of the enzyme (3, 12). Under conditions optimal for the stabilization of the E_1—P form of the enzyme its reactivity with ouabain appears to be markedly reduced, supporting earlier observations and suggestions that the Na^+-stimulated pathway of [^{3}H]ouabain binding requires the formation of phospho-enzyme (12–14).

[1] The terms E—P and phospho-enzyme are used interchangeably throughout this paper to indicate phosphorylation of $(Na^+ + K^+)$-ATPase, without any distinction being made as to the form of phospho-enzyme present.

[2] R. L. Post, personal communication.

A preliminary report has been published (15).

MATERIALS AND METHODS

Enzyme preparation and assay. Rat brain ($Na^+ + K^+$)-ATPase was prepared as described by Akera and Brody (16). Its ($Na^+ + K^+$)-ATPase activity was assayed by a modification of the method of Post and Sen (17), and its protein content was estimated by the method of Lowry *et al.* (18). Total ATPase activity varied between 150 and 300 μmoles of P_i per milligram of protein per hour, and about 90–95% of the activity was ouabain-sensitive.

The modification of the assay system of Post and Sen (17) was the method for P_i determination. After the enzymatic reaction had been stopped by the addition of perchloric acid and sodium molybdate had been added, the phosphomolybdate complex was extracted into 5 ml of butyl acetate as previously described (17). The absorbance of the phosphomolybdate complex was then estimated directly at 410 nm. The results obtained in this way were similar to those obtained by the original method of Post and Sen (17) or by the method of Akera and Brody (16).

Phosphorylation of ($Na^+ + K^+$)-ATPase. The ($Na^+ + K^+$)-ATPase was phosphorylated by a modification of the method of Post and Sen (19). All reactions were carried out in 50 mM Tris buffer, pH 7.4, at 0°, unless otherwise noted, and in a final reaction volume of 1.0 ml. The reaction system contained 90–150 μg of enzyme protein, and the standard ionic conditions were 100 mM Na^+, 1 mM Mg^{++}, and 0.05 mM [γ-^{32}P]ATP. The labeling reaction was usually started by the rapid addition of 0.1 ml of [γ-^{32}P]ATP from a 1.0-ml tuberculin syringe to an otherwise complete system. Subsequent additions were made similarly in 0.1-ml volumes. The labeling reaction was terminated by the rapid injection of 4 ml of an ice-cold 5% trichloracetic acid solution containing 1 mM each unlabeled ATP and P_i. The reaction tubes were then held on ice for up to 30 min before filtering.

Filtering was performed on 24-mm-diameter Millipore filters (0.8-μ pore diameter) as described by Chignell and Titus (20). The filters were first washed with 4 ml of the trichloracetic acid–ATP–P_i solution used to stop the phosphorylation reaction. The acid-precipitated enzyme was then placed on the filter and washed four times with 4-ml aliquots of the ice-cold trichloracetic acid–ATP–P_i solution. The filters were transferred to scintillation vials, and 10 ml of Bray's scintillation medium were added. Counting was performed in a Beckman LSE 100 liquid scintillation spectrophotometer, and picomoles of ^{32}P per milligram of protein were calculated by the method of Post and Sen (19). Labeling was calculated on the basis of the amount of protein added to the reaction system, and no correction was made for possible loss of protein, which appeared to be minimal (20, 21).

To allow comparison of experiments performed with different enzymes, the amount of phospho-enzyme formed in the presence of 100 mM Na^+, 1 mM Mg^{++}, and 0.5 mM [γ-^{32}P]ATP was arbitrarily set at 100% (22) and other values were expressed as a percentage of this. Labeling in the presence of 16 mM K^+ plus Mg^{++} or Ca^{++} and [γ-^{32}P]-ATP was deducted as background unless otherwise stated. The specific (sodium-stimulated) incorporation of ^{32}P was between 250 and 500 pmoles of ^{32}P per milligram of protein.

Binding of [^{3}H]ouabain. Binding of [^{3}H]-ouabain was performed by the method of Matsui and Schwartz (14) as described by Tobin and Sen (12). All experiments were performed in 50 mM Tris buffer, pH 7.4, at 37° in a final reaction volume of 1 ml. The reaction mixture contained about 200 μg of enzyme protein, and the other conditions were as noted in the figure legends. The binding reaction was terminated by the addition of 4 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 250 μM unlabeled ouabain. This addition served to cool the reaction medium and prevent the further binding of [^{3}H]ouabain (23). The reaction tubes were then placed on ice and allowed to cool at 0°, at which temperature the enzyme-ouabain complex is relatively stable (12, 13). After cooling, the reaction tubes were centrifuged at 40,000 × *g* for 15 min

to sediment the microsomal fraction, the supernatant fluid was discarded, and the sediment was solubilized and counted as described previously (12). [^{3}H]Ouabain binding is expressed as picomoles of [^{3}H]ouabain per milligram of protein; the protein value was taken as the amount added to the binding system. To allow comparison of experiments with different enzymes, the highest value was arbitrarily set at 100% and the other values were expressed as a percentage of this (24).

Reagents and chemicals. [γ-^{32}P]ATP was obtained from New England Nuclear Corporation and was diluted with carrier ATP to give about 5×10^6 cpm/μmole of ATP. [^{3}H]Ouabain (New England Nuclear) was diluted with carrier ouabain to give 500 Ci/mole, the high specific activity being required by the relatively low concentration of ouabain used in the initial rate of binding experiments (24). The carrier ATP, ouabain, and other chemicals were purchased from Sigma Chemical Company. All Ca^{++} concentrations are those added to the medium, and no attempt was made to calculate the concentration of free Ca^{++} under the various experimental conditions. All experiments were repeated at least four times, and the data presented are the means of at least four experimental determinations on different enzyme preparations, plus or minus standard errors of the means. Lack of a vertical bar indicates that the measured standard error was less than 2%. Where appropriate, statistical significance was calculated by the *t*-test, the criterion for significance being $p < 0.05$. Hofstee plots were used to determine the types of inhibition (25).

RESULTS

Actions of Ca^{++} on turnover of $(Na^+ + K^+)$-ATPase. Figure 2 shows the action of increasing concentrations of Ca^{++} on the activity of rat brain $(Na^+ + K^+)$-ATPase (solid circles). With increasing Ca^{++} concentrations $(Na^+ + K^+)$-ATPase activity declined, and the concentration for half-maximal inhibition by Ca^{++} was about 0.5 mM, essentially similar to the observations of other investigators (6–8, 26, 27). Values

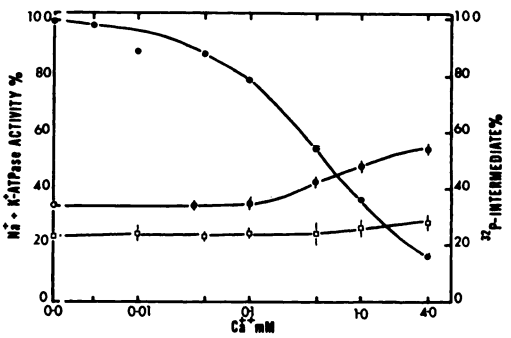

FIG. 2. *Ca^{++} inhibition of $(Na^+ + K^+)$-ATPase activity and its effect on steady-state levels of E—P*
●——●, inhibition of $(Na^+ + K^+)$-ATPase under standard conditions (100 mM Na^+, 16 mM KCl, 5 mM MgATP, and 20 μg of enzyme protein; pH 7.4; 37°) by the addition of the indicated concentrations of Ca^{++}; □——□, steady-state levels of *E*—P observed under similar ionic conditions at 0°; ○——○, steady-state levels of *E*—P observed in the presence of 100 mM Na^+, 3 mM K^+, 0.4 mM Mg^{++}, and the indicated concentrations of Ca^{++}. This concentration of Mg^{++} was sufficient to produce 100% *E*—P in the absence of K^+. At 4 mM Ca^{++} the steady-state level of *E*—P is significantly greater than in its absence. The steady-state levels of *E*—P are plotted as a percentage of the level observed in the presence of 100 mM Na^+, 1 mM Mg^{++}, and 0.05 mM [γ-^{32}P]ATP, with the labeling observed when 16 mM K^+ was substituted for the Na^+ deducted as background labeling. The "100%" $(Na^+ + K^+)$-ATPase activity was 162 μmoles of P_i per milligram of protein per hour.

designated by the open squares show the lack of effect of Ca^{++} on the steady-state levels of *E*—P under similar ionic conditions. These latter experiments were conducted at 0° and at the concentrations of [γ-^{32}P]ATP used in the phosphorylation experiments. Although these experiments did not support the concept of Ca^{++} inhibition of the $E\text{—P} \rightleftharpoons E_2\text{—P}$ transformation, the data show a trend toward higher steady-state levels of *E*—P at higher Ca^{++} concentrations. Similar experiments, in the presence of lower Mg^{++} concentrations (0.4 mM), however, showed a significant increase in the steady-state levels of *E*—P as the concentration of Ca^{++} was increased.

This increase in the steady-state level of *E*—P could be due to an effect of Ca^{++} on the E_1—P to E_2—P transformation or to an

TABLE 1

Phospho-enzyme disappearance in the presence and absence of Ca^{++}

$(Na^+ + K^+)$-ATPase was labeled with 0.05 mM [γ-^{32}P]ATP in the presence of 100 mM Na^+ and 1 mM Mg^{++}. Six seconds after the addition of the [γ-^{32}P]ATP, 1 mM unlabeled ATP or 1 mM ATP plus 1 mM Ca^{++} were added and the reaction was stopped at the time intervals indicated. The amount of phospho-enzyme observed is expressed as a percentage of that observed at 6 sec, which was 535.56 ± 20.71 pmoles of ^{32}P per milligram of protein. No deductions for background labeling were made.

Time	Phospho-enzyme	
	$-Ca^{++}+$	$+Ca^{++}$
sec	%	%
0	100	100
3	55.6 ± 1.1	53.0 ± 3.1
6	37.7 ± 2.3	41.6 ± 2.8
9	36.4 ± 1.8	32.3 ± 1.1
12	31.4 ± 2.9	28.3 ± 0.9

action of Ca^{++} on the breakdown of E_2—P. Table 1 shows the lack of effect of Ca^{++} on the spontaneous hydrolysis of E_2—P, which is considered to be due to the presence of residual K^+ (28). The results suggest that Ca^{++} does not inhibit the breakdown of E_2—P, indicating that Ca^{++} acts to increase the steady-state levels of E—P prior to the formation of E_2—P. Because of the relatively rapid turnover of E_2—P in the rat brain enzyme it was not feasible to test the effect of a significant concentration of added K^+ (11, 24).

Figure 3 shows the effects of Ca^{++} on the steady-state levels of E—P (Fig. 6). Under "standard" phosphorylation conditions (100 mM Na^+, 5 mM Mg^{++}, and 0.05 mM [γ-^{32}P]-ATP) Ca^{++} had little effect on the steady-state level of E—P. Furthermore, if Mg^{++} was omitted from the incubation medium, Ca^{++} alone produced a substantial increment in labeling. This Ca^{++}-stimulated formation of phospho-enzyme agrees with the recent observations of Blostein and Burt (8) and Shamoo and Brodsky (29) and weakens suggestions that the action of Ca^{++} is simply to inhibit the formation of E—P (30).

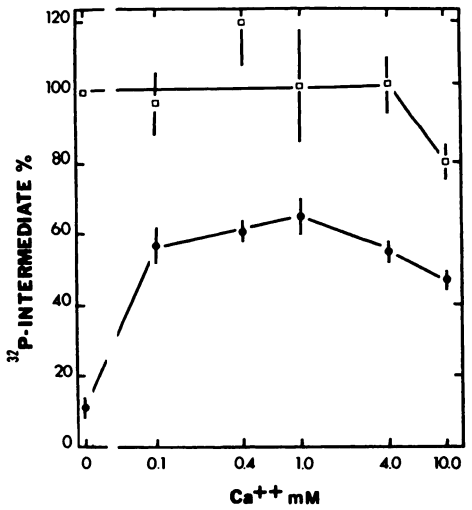

FIG. 3. *Effect of Ca^{++} on steady-state level of E—P in the absence of K^+*

□——□, amount of E—P formed after 5 sec in the presence of 100 mM Na^+, 5 mM Mg^{++}, 0.05 mM [γ-^{32}P]ATP, and the indicated concentrations of Ca^{++} at 0°. Labeling in the absence of added Na^+ was deducted as a background, and the Na^+-stimulated increment in labeling averaged 451.0 pmoles of ^{32}P per milligram of protein. ●——●, Na^+-stimulated increment in labeling at the indicated Ca^{++} concentrations when no Mg^{++} was added.

Since these membrane preparations are relatively impure, it was mandatory to test the Ca^{++}-stimulated increment in phosphorylation in such a way that it could be determined that this effect involved the $(Na^+ + K^+)$-ATPase. Table 2 shows that Ca^{++} alone did not stimulate the labeling of these membrane preparations above background levels, but that Na^+ and Ca^{++} gave rise to 84% of the maximal amount of E—P. Other experiments on the time course of phosphorylation showed that Na^+- and Ca^{++}-dependent labeling did not increase after 3 sec (see also Fig. 6). K^+ was unable to stimulate phosphorylation in the presence of Ca^{++} and was also relatively ineffective in reducing the labeling, as shown by the 47% of phospho-enzyme observed in the presence of Na^+ and K^+ (condition 6). More significantly, if ouabain was allowed to bind to the enzyme during preliminary incubation at 0°, the Na^+- and Ca^{++}-stimulated labeling was completely inhibited

TABLE 2

Cation and glycoside sensitivity of Ca^{++}-dependent labeling of rat brain $(Na^+ + K^+)$-ATPase

Rat brain enzyme was incubated with 0.05 mM [γ-^{32}P]ATP in the presence of the indicated ligands for 10 sec at 0°. The ^{32}P trapped on the Millipore filter was calculated as a percentage of that trapped on the filter after labeling in the presence of Na^+ and Mg^{++} (464.5 ± 10.9 pmoles of ^{32}P per milligram of protein). The cation concentrations were: Na^+, 100 mM; K^+, 16 mM; Mg^{++} and Ca^{++}, 1 mM each. For the experiments with ouabain the enzyme was incubated with 250 μM ouabain overnight at 0° in the presence and absence of 100 mM Na^+. When Na^+ was absent overnight it was added 5 min before the labeling reaction was started.

Ionic conditions	^{32}P labeling of membranes
	%
1. $Na^+ + Mg^{++}$	100 ± 0
2. $K^+ + Mg^{++}$	7.5 ± 1.2
3. Ca^{++}	9.5 ± 1.84
4. $Na^+ + Ca^{++}$	84.7 ± 3.7
5. $K^+ + Ca^{++}$	7.1 ± 2.0
6. $Na^+ + Ca^{++} + K^+$	47.6 ± 3.3
7. Na^+, Ca^{++}, ouabain[a]	72.7 ± 4.5
8. Ouabain, Ca^{++}, Na^+[b]	12.8 ± 1.1

[a] Na^+ was present during overnight incubation.
[b] Na^+ was absent during overnight incubation.

(condition 8). However, if binding of ouabain was prevented by the presence of Na^+ in the preliminary incubation medium (22), the inhibition of labeling was minimal (condition 7). These results suggest that the Ca^{++}-stimulated labeling of these membrane preparations involves $(Na^+ + K^+)$-ATPase and that Ca^{++} can at least partially substitute for Mg^{++} in the reaction mechanism of this enzyme.

Since Fig. 2 shows that under standard conditions for the assay of $(Na^+ + K^+)$-ATPase (100 mM Na^+, 5 mM MgATP, and 15 mM K^+) the major effect of Ca^{++} is not to prevent the breakdown of E—P, we studied the interaction of Ca^{++} with Na^+ and Mg^{++}, the two cofactors required for the transphosphorylation reaction (Fig. 1, reaction 1). Figure 4 shows the effects of Ca^{++} on the Na^+-stimulated formation of phospho-enzyme. In the absence of Ca^{++} the apparent K_m for Na^+ for phosphorylation is on the order of 1 mM, but the addition of Ca^{++} reduces the apparent affinity of the enzyme for Na^+ to about 30 mM. From a Hofstee plot of these data the interaction between Na^+ and Ca^{++} appears to be competitive. In contrast, Ca^{++} alone appears to be only partially effective in stimulating the formation of E—P. The similarity of the Ca^{++}- and $(Ca^{++} + Mg^{++})$-dependent labeling suggests that in the presence of combinations of these ions the dominant divalent cation is Ca^{++}.

The apparently parallel Mg^{++} and $(Mg^{++} + Ca^{++})$ plots of Fig. 4 suggest that the interaction between Mg^{++} and Ca^{++} is noncompetitive. Since this contrasts sharply with a previous report (7), we investigated the action of 0.5 mM Ca^{++} on the Mg^{++} activation of this enzyme at 37° (Fig. 5). Although the interaction is complex at concentrations above 3 mM Mg^{++} (at which concentrations Mg^{++} becomes inhibitory), it is noncompetitive below 2 mM Mg^{++}, as indicated by the parallel lines yielded by the Hofstee plot (right-hand panel, Fig. 5).

These observations indicate that Ca^{++} interferes with the ability of both Na^+ and Mg^{++} to activate their respective steps in the $(Na^+ + K^+)$-ATPase reaction, but that Ca^{++} nevertheless must be present at very high concentrations to reduce the steady-state levels of E—P (Fig. 3). Presumably Ca^{++} inhibits the initial rate of the phosphorylation reaction, but substantial inhibition of the initial rate of phosphorylation is required to reduce the steady-state levels of E—P. We therefore tested the actions of Ca^{++} on the initial rate of formation of phospho-enzyme. Because the initial rate of phosphorylation of the guinea pig kidney $(Na^+ + K^+)$-ATPase is slowest when the cations and [γ-^{32}P]ATP are added together,[3] this labeling method was chosen. Figure 6 shows the initial rates of formation of phospho-enzyme in the presence and absence of Ca^{++}. With Mg^{++} alone labeling reached a steady state within 1 sec. With Mg^{++} and Ca^{++} labeling was deficient at 1 sec and did not increase significantly after 3 sec. With

[3] Unpublished observations.

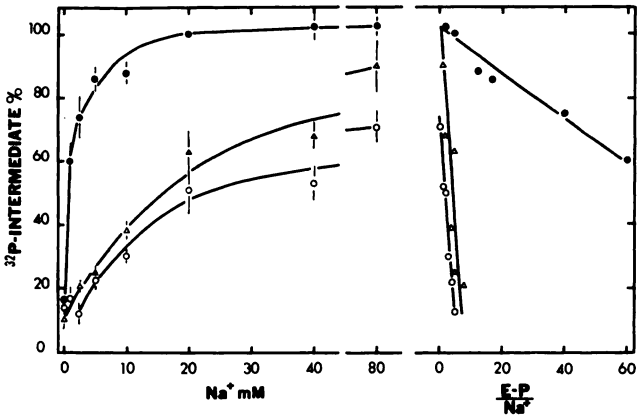

FIG. 4. *Na^+ stimulation of E—P formation in the presence of Mg^{++}, Ca^{++}, and Mg^+ plus Ca^{++}*

●——●, Na^+-stimulated increment in labeling in the presence of 1 mM Mg^{++}, 0.05 mM [γ-^{32}P]ATP, and the indicated concentrations of Na^+. The concentrations of Na^+ plotted are those added to the system, which presumably contained residual Na^+ to account for phosphorylation in the absence of added Na^+. ○——○, labeling when Ca^{++} was substituted for Mg^{++}; △——△, labeling when Mg^{++} and Ca^{++} were combined. The right-hand panel shows these data plotted according to the method of Hofstee (25). Labeling in the presence of 20 mM Na^+ and 1 mM Mg^{++}, 542 ± 48 pmoles of ^{32}P per milligram of protein, was taken as 100% *E*—P, and other values were plotted as a percentage of it.

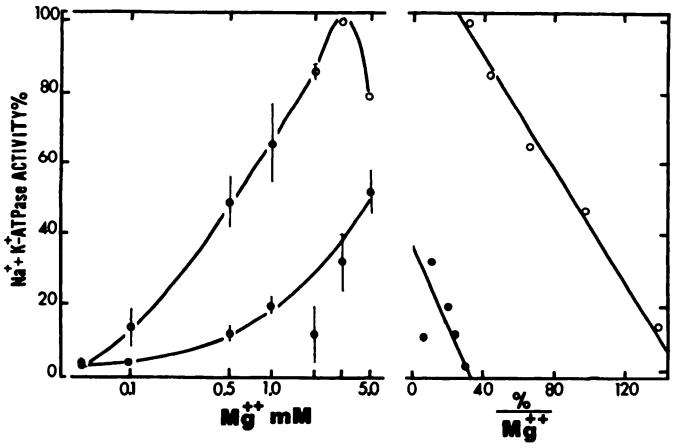

FIG. 5. *Noncompetitive interaction between Mg^{++} and Ca^{++}*

The activity of 15 μg of enzyme protein was assayed in the presence of 100 mM Na^+, 16 mM K^+, and 5 mM ATP, with the Mg^{++} concentration varied as indicated on the horizontal axis. ○——○, enzyme activity in the absence of added Ca^{++}; ●——●, activity in the presence of 0.5 mM Ca^{++}. The right-hand panel shows these data plotted by the method of Hofstee (25), and the fitted lines are orthogonal regressions. Data are plotted as a percentage of the activity with 3 mM Mg^{++}, 184 μmoles of P_i per milligram of protein per hour.

Ca^{++} alone labeling was markedly deficient at 1 sec but again had reached a steady-state level at 3 sec. The data show that Ca^{++} alone stimulates a very slow phosphorylation of this enzyme and that Ca^{++} also acts to slow the initial rate of phosphorylation in the presence of Mg^{++}.

Figure 7 shows the effects of increasing concentrations of K^+ on the steady-state levels of *E*—P formed in the presence of sodium and Mg^{++}, Ca^{++}, or Mg^{++} plus Ca^{++}. If the phospho-enzyme is formed in the presence of 16 mM Na^+ and 1 mM Mg^{++}, 10 mM K^+ is able to reduce the steady-state

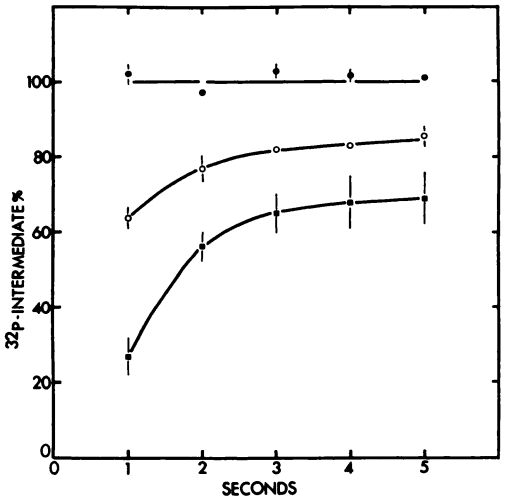

FIG. 6. *Rates of formation of E—P in the presence of Mg^{++}, Ca^{++}, and Mg^{++} plus Ca^{++}*

Rat brain enzyme was incubated in 50 mM Tris-HCl, pH 7.4, at 0°. At zero time 100 mM Na^+, 0.05 mM [γ-^{32}P]ATP; either 1 mM Mg^{++} (●——●), 1 mM Ca^{++} (■——■), or 1 mM Mg^{++} plus 1 mM Ca^{++} (○——○) was added, and the labeling reaction was stopped at the indicated time points. Labeling in the presence of Na^+, Mg^{++}, and [γ-^{32}P]-ATP at 5 sec (610.0 pmoles of ^{32}P per milligram of protein) was taken as 100%, and labeling with 10 mM K^+ substituted for Na^+ was deducted as background labeling. Labeling in the presence of Mg^{++} and Ca^{++} was significantly ($p < 0.01$) lower at 1 sec than at 5 sec.

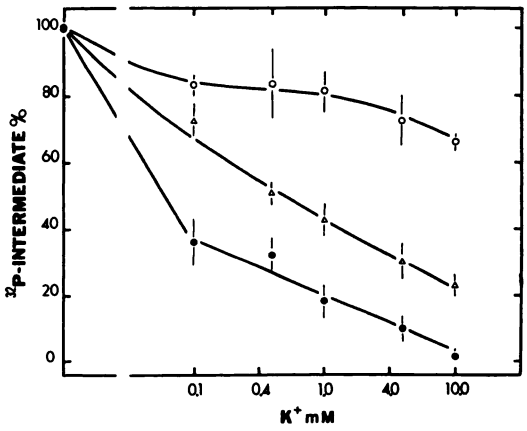

FIG. 7. *Sensitivity of steady-state levels of E—P in the presence of Mg^{++} and Ca^{++} to varied concentrations of K^+*

Each point is the steady-state level of phospho-enzyme observed at the indicated concentrations of K^+ 5 sec after the addition of [γ-^{32}P]ATP. Labeling in each case is plotted as a percentage of that observed in the absence of added K^+, with labeling in the presence of K^+, Mg^{++}, and [^{32}P]-ATP deducted as background. ●——●, labeling observed in the presence of 16 mM Na^+, 1 mM Mg^{++}, and the indicated K^+ concentrations; ●——●, labeling observed in the presence of 100 mM Na^+, 1 mM Ca^{++}, and the indicated concentrations of K^+; △——△, labeling under the same conditions with 1 mM Mg^{++} added. Labeling was 436.93 ± 47.03 pmoles of ^{32}P per milligram of protein in the presence of Mg^{++}, 314.64 pmoles/mg in the presence of Ca^{++}, and 334.56 pmoles/mg when the cations were combined.

level of *E*—P to about the amount of labeling observed in the presence of K^+, Mg^{++}, and [γ-^{32}P]ATP. In the presence of 100 mM Na^+, 1 mM Mg^{++}, and 1 mM Ca^{++}, 10 mM K^+ reduced the steady-state level of *E*—P to about that observed in the presence of Mg^{++} alone (cf. Fig. 2). However, the *E*—P formed in the presence of Ca^{++} alone was relatively resistant to K^+; 10 mM K^+ reduced the steady-state level of *E*—P to only about 65% of that observed in the absence of added K^+. This insensitivity of the Ca^{++}-dependent phospho-enzyme to K^+ is remarkable because the relatively slow Ca^{++}-dependent formation of *E*—P (Fig. 6) should render the steady-state level of *E*—P more sensitive to factors which accelerate its breakdown.

Properties of the Ca^{++}-dependent phospho-enzyme. One of the hypotheses on which this work was based was the possibility that Ca^{++} might inhibit the E_1—P $\rightleftharpoons$ E_2—P transformation. We studied the actions of a number of agents on the E_1—P to E_2—P transformation and were able to demonstrate the partial reversal of this step.

Classically, the phospho-enzyme formed in the presence of Na^+, Mg^{++}, and ATP reacts readily with K^+ and not at all with ADP, and the E_1—P form is only recognized in enzymes irreversibly inhibited with NEM[4] (3). However, it appears relatively easy to demonstrate an ADP-sensitive intermediate in rat brain $(Na^+ + K^+)$-ATPase, as shown in Fig. 8. When the phospho-enzyme is formed in the presence of 12 mM Na^+ and

[4] The abbreviations used are: NEM, *N*-ethylmaleimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

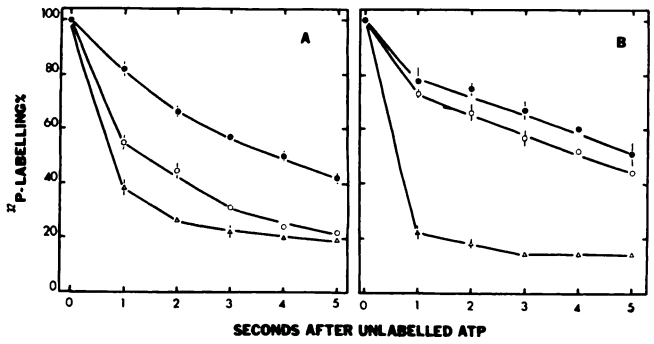

FIG. 8. *Reactivity of E—P with ADP: effects of high Na^+ concentrations*

A. Rat brain (Na^+ + K^+)-ATPase was phosphorylated in the presence of 120 mM Na^+, 1 mM Mg^{++}, and 0.05 mM [γ-^{32}P]ATP. Five seconds after the addition of the [γ-^{32}P]ATP the following additions were made at zero time, and the reaction was stopped at the time points indicated: ●——●, unlabeled ATP (final concentration, 1 mM); ○——○, unlabeled ATP (1 mM) plus ADP (0.1 mM); △——△, unlabeled ATP plus KCl (1 mM each). The data are plotted as a percentage of the amount of labeling at 5sec (672.73 ± 53.71 pmoles of ^{32}P per milligram of protein), with no deductions for background labeling. One second after the addition of unlabeled ATP ± ADP the amount of phospho-enzyme was reduced significantly from that seen in the presence of ATP alone ($p < 0.05$).

B. Duplicate experiments, in which the Na^+ concentration was held at 12 mM. All other conditions and enzyme preparations were the same as in Fig. 8A.

tested for its reactivity with ADP or K^+ (Fig. 8B), it shows a typical E_2—P pattern of high K^+ and low ADP sensitivity (3). However, if this experiment is repeated in the presence of 120 mM Na^+, the reactivity of the phospho-enzyme with ADP is markedly increased. In other experiments, in which the enzyme was phosphorylated in the presence of 12 mM Na^+ and 140 mM Na^+ was added with the ADP, a similar increased reactivity with ADP (15) was observed. Thus it appears that in the presence of high Na^+ concentrations the Mg^{++}-dependent phospho-enzyme becomes reactive with ADP.

This effect of Na^+ is only partial, however, and does not result in the formation of a K^+-insensitive intermediate. The data of Fig. 7, which show that the Ca^{++}-dependent phospho-enzyme is resistant to K^+, prompted us to test the effects of ADP on the Ca^{++}-dependent phospho-enzyme. Figure 9 shows that if the phospho-enzyme is formed in the presence of Ca^{++}, its reactivity is considerably altered. It now reacts preferentially with ADP and poorly with K^+. In this particular experiment (Fig. 9) the concentration of ADP was increased (to 1 mM) to show that essentially all the phos-

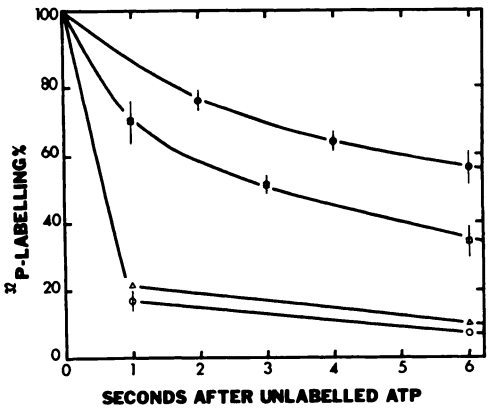

FIG. 9. *Reactivity of Ca^{++}-dependent phospho-enzyme with ADP and K^+*

The enzyme was phosphorylated in the presence of 120 mM Na^+ and 1 mM Ca^{++} with 0.05 mM [γ-^{32}]-ATP as before. At 5 sec, indicated as zero time, the following additions were made: 1 mM ATP alone (●——●) or combined with 1 mM ADP (△——△) or with 1 mM KCl (□——□). The symbols indicate the amounts of labeling remaining at the indicated time points. ○——○, time course of labeling after the simultaneous addition of 1 mM ATP, 1 mM ADP, and 16 mM KCl. The data are expressed as a percentage of the amount of labeling observed at 5 sec (480.06 ± 20.3 pmoles of ^{32}P per milligram of protein), with no deductions for background labeling.

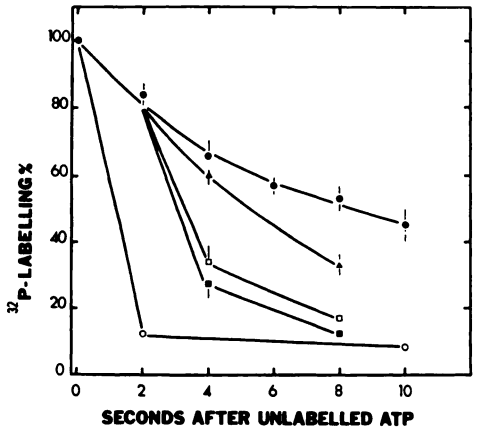

FIG. 10. *Reactivity of Ca^{++}-dependent E—P with ADP: effects of Mg^{++} plus EGTA*

The $(Na^+ + K^+)$-ATPase was phosphorylated with 0.05 mM [γ-^{32}P]ATP in the presence of 100 mM Na^+ and 0.4 mM Ca^{++}. After 5 sec, indicated as zero time, 1 mM unlabeled ATP was added. ●——●, amount of labeling observed at the indicated time points. ○——○, amount of labeling observed at 2 and 10 sec after the addition of 1 mM ATP, 1 mM ADP, and 16 mM K^+. Two seconds after zero time the reactivity of the phospho-enzyme with the following combinations of ligands was tested: ADP, 0.1 mM (■——■); ADP, 0.1 mM; EGTA, 5 mM; and Mg^{++}, 10 mM (▲—▲); and ADP, 1 mM; EGTA, 5 mM; and Mg^{++}, 10 mM (□——□). Labeling is expressed as a percentage of the amount of labeling present at zero time (395.25 ± 54 pmoles of ^{32}P per milligram of protein), with no background values deducted. In other experiments the addition of Mg^{++} and EGTA did not significantly alter the rate of turnover of *E*—P or the levels of background labeling.

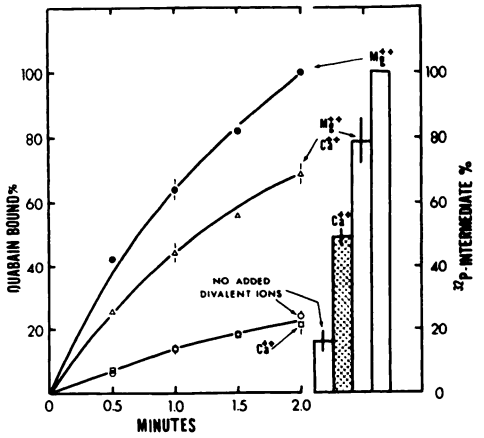

FIG. 11. *Effects of Ca^{++} on steady-state levels of E—P and initial rates of [^{3}H]ouabain binding*

The left-hand panel shows the initial rates of [^{3}H]ouabain binding at 37° in the presence of 200 mM Na^+, 3 mM ATP, and 50 nM [^{3}H]ouabain, with divalent cations as follows: ○——○, no added divalent cations; □——□, 1 mM Ca^{++}; ●——●, 1 mM Mg^{++}; △——△, 1 mM Mg^{++} plus 1 mM Ca^{++}. All values are plotted as a percentage of the labeling with 1 mM Mg^{++} at 2 min, which averaged 112.8 ± 9.8 pmoles of [^{3}H]ouabain per milligram of protein. The right-hand panel shows the steady-state levels of phospho-enzyme formed at 5 sec under similar conditions at 37°, expressed as a percentage of that formed with 1 mM Mg^{++} (427.6 ± 34 pmoles of ^{32}P per milligram of protein). The only difference between the two experiments is the lower (0.05 mM) [γ-^{32}P]ATP concentration used for the phosphorylation studies.

pho-enzyme formed would react with ADP. This altered reactivity is very similar to the reactivity of NEM-treated phospho-enzymes (3).

The E_1—P to E_2—P change is usually considered to require Mg^{++} (3, 9). Figure 10 shows some direct evidence in support of this hypothesis. As in previous experiments, the phospho-enzyme formed in the presence of Ca^{++} reacted with ADP, but when EGTA plus excess Mg^{++} was added, the reactivity of the phospho-enzyme with ADP was markedly reduced. This observation is consistent with the hypothesis that Mg^{++} allows the E_1—P to E_2—P transformation to proceed and that the E_2—P configuration is less reactive with ADP (9). However, the effect of Mg^{++} was only partial, in that it did not increase the reactivity of the phospho-enzyme with K^+ and that increasing the concentration of ADP overcame the effects of EDTA plus Mg^{++}.

In a number of other experiments the reactivity of the Ca^{++}-dependent phospho-enzyme with hydroxylamine, CDP, and IDP was tested. None of these agents produced a significant increase in the rate of dephosphorylation of the Ca^{++}-dependent phospho-enzyme.

Effects of Ca^{++} on binding of [^{3}H]ouabain to $(Na^+ + K^+)$-ATPase. The observation that Ca^{++} stimulated the formation of a K^+-resistant phospho-enzyme prompted us to test the reactivity of the Ca^{++}-dependent phospho-enzyme with ouabain. In Fig. 11

we compared the initial rates of binding of [^{3}H]ouabain with the steady-state levels of E—P formed under similar conditions at 37°. The concentration of Na^+ was 200 mM, both because of the ability of high Na^+ to favor the E_1—P conformation (Fig. 8) and because this concentration of Na^+ favors the binding of [^{3}H]ouabain to guinea pig kidney phospho-enzyme (12, 22). The initial rates of [^{3}H]ouabain binding and steady-state levels of E—P occurring in the presence of Na^+ and Mg^{++} were arbitrarily set at 100%. The addition of Ca^{++} in the presence of Mg^{++} produced a small equivalent inhibition of both [^{3}H]ouabain binding and E—P formation. Similarly, in the absence of added divalent cations, low (and equivalent) levels of both phosphorylation and [^{3}H]ouabain binding were observed. However, the addition of Ca^{++} alone to the system produced a 3-fold increment in the formation of phospho-enzyme without any comparable increase in the rate of [^{3}H]ouabain binding (see also ref. 31). The data suggest that the Ca^{++}-dependent form of the phospho-enzyme reacts poorly with ouabain, while the phospho-enzyme formed in the presence of Mg^{++} and Ca^{++} is fully sensitive to ouabain.

Table 3 shows the actions of Ca^{++} on the Na^+-inhibited pathway of [^{3}H]ouabain binding (12). Ca^{++} alone or in the presence of P_i poorly stimulates [^{3}H]ouabain binding and is an effective inhibitor of Mg^{++}- and P_i-dependent binding. Similarly, Ca^{++} is considerably less effective than Mg^{++} in supporting binding in the presence of either ATP or ADP, and the addition of Na^+ results in little change in Ca^{++}- and ADP-dependent binding. This observation is consistent with the hypothesis that Ca^{++} interferes with the Mg^{++}-stimulated $E_1 \rightleftharpoons E_2$ transformation (12, 22) and that NaE_1ADP is not a ouabain-binding species (but see ref. 32).

TABLE 3

Effects of Ca^{++} on Na^+-inhibited pathway of [^{3}H]-ouabain binding

Rat brain (Na^+ + K^+)-ATPase was incubated with 50 nM [^{3}H]ouabain in the presence of the indicated ligands at 37° for 6 min. Binding of [^{3}H]ouabain, in picomoles of [^{3}H] ouabain per milligram of protein, is expressed as a percentage of that found in the presence of Mg^{++} and P_i, which averaged 140.7 ± 5.4 pmoles/mg of protein.

Additions	[^{3}H]Ouabain bound
	%
None	1.83 ± 0.2
Ca^{++}, 1 mM	2.37 ± 0.17
Mg^{++}, 1 mM	4.64 ± 0.47
Ca^{++}, 1 mM; P_i, 1 mM	8.44 ± 0.47
Ca^{++}, 2 mM; ADP, 2 mM	3.85 ± 0.36
Ca^{++}, 2 mM; ADP, 2 mM; Na^+, 100 mM	6.04 ± 0.64
Ca^{++}, 2 mM; ATP 2 mM	11.6 ± 1.80
Ca^{++}, 1 mM; ATP, 2 mM; Mg^{++}, 2 mM	76.68 ± 4.63
ATP, 2 mM; Mg^{++}, 2 mM	81.75 ± 1.96
Ca^{++}, 1 mM; Mg^{++}, 1 mM; P_i, 1 mM	49.48 ± 0.82
Mg^{++}, 1 mM; P_i, 1 mM	100

DISCUSSION

The primary action of Ca^{++} in inhibiting the turnover of (Na^+ + K^+)-ATPase is on the rate of formation of E—P. Inhibition at step 2 or 3 (Fig. 1) would give rise to higher steady-state levels of E—P, and effect observed only at low concentrations of Mg^{++} (Fig. 2). In the presence of Mg^{++} and Ca^{++} the phospho-enzyme formed is sensitive to K^+, dephosphorylation occurs, and the steady-state level of E—P remains low. The ability of Ca^{++} alone to stimulate the formation of phospho-enzyme shows a direct action of Ca^{++} on transphosphorylation, but the mechanism by which Ca^{++} inhibits the formation of E—P remains unclear, since either reaction 1 or 4 may be the primary site of action of Ca^{++}. While this work was in progress a number of reports appeared confirming these observations (8, 27, 29) and one earlier observation (11) concerning the Ca^{++}-stimulated formation of the phospho-enzyme. These observations provide evidence against the hypothesis that Ca^{++} simply acts to inhibit the formation of the phospho-enzyme (30).

The attempts to elucidate the mechanism by which Ca^{++} inhibits the phosphorylation step were only partially successful. The interaction with Mg^{++} appears to be noncompetitive (Fig. 5). This observation is in good agreement with the data of Skou (26),

who showed that in the presence of Ca^{++} increasing Mg^{++} concentrations were unable to overcome the inhibitory actions of Ca^{++}. In contrast, Epstein and Whittam (7) considered that CaATP acted as a competitive inhibitor of MgATP, the proposed substrate for $(Na^+ + K^+)$-ATPase. However, recent evidence has shown that MgATP is not necessarily the only substrate of $(Na^+ + K^+)$-ATPase (3, 33), and the data presented by Epstein and Whittam (7) appear more complex than the double-reciprocal treatment of the data suggests.

The apparently strictly competitive interaction between Na^+ and Ca^{++} for the formation of E—P at 0° agrees well with earlier reports. Blostein and Burt (8) observed that chelation of residual Ca^{++} with EGTA increased the apparent affinity of red cell $(Na^+ + K^+)$-ATPase for Na^+, and Portius and Repke (34) have reported that increasing Na^+ concentrations overcome the inhibitory effects of Ca^{++} on the $(Na^+ + K^+)$-ATPase. However, other experiments in this laboratory on the interaction of Ca^{++} and Na^+ on this enzyme at 37° indicated that the Ca^{++} inhibition of the over-all $(Na^+ + K^+)$-ATPase activity under standard conditions was noncompetitive with respect to Na^+, in agreement with the data of Epstein and Whittam (7). Figure 6, which shows that Ca^{++} substitutes poorly for Mg^{++} in the transphosphorylation reaction, is about as far as the mechanism of the Ca^{++}-dependent inhibition of the phosphorylation step can be pursued with the techniques used here (35).

The observation that Ca^{++} can stimulate the labeling of these membranes by [γ-^{32}P]-ATP in the absence of added Mg^{++} agrees with the observations of other workers (8, 11, 27, 29), and its inhibition by bound ouabin supports the hypothesis that this labeling occurs to the $(Na^+ + K^+)$-ATPase. The possibility that Ca^{++} acts simply by displacing bound Mg^{++}, which then activates the phosphorylation step, remains. In a number of experiments attempts were made to remove residual Mg^{++} by dialyzing the enzyme against 1 mM EDTA, but such treatment did not reduce the amount of Ca^{++}-dependent phospho-enzyme formed.

Of greater interest are the properties of the E_2—P form of the rat brain phospho-enzyme and the Ca^{++}-dependent phospho-enzyme. The E_2—P form of guinea pig kidney $(Na^+ + K^+)$-ATPase is almost completely insensitive to ADP, and in this tissue the E_1—P to E_2—P transformation is considered irreversible (3). In contrast, the rat brain phospho-enzyme reacts readily with ADP in the presence of high concentrations of Na^+, although there is little change in the reactivity of the system with K^+. As indicated under RESULTS, the same effect is obtained if Na^+ is added with ADP, i.e., after the formation of the Mg^{++}-dependent E_2—P intermediate (see ref. 15). These experiments suggest that in the rat brain enzyme the E_1—P $\rightleftharpoons$ E_2—P equilibrium is reversible and that high concentrations of Na^+ favor the E_1—P form. Na^+, which cooperatively stabilizes the E_1 form of the native $(Na^+ + K^+)$-ATPase (12), presumably also tends to stabilize the same configuration of the phospho-enzyme, but less effectively in this case. Phosphorylation, by favoring the E_2 configuration, reduces the affinity of the enzyme for Na^+, and this change in affinity is presumably associated with the transport of Na^+. These actions of Na^+ on the E_1—P $\rightleftharpoons$ E_2—P equilibrium support the inclusion of Na^+ in Eq. 2 of Fig. 1. The reversibility of this reaction, and the recent demonstration by Toda *et al.* (36) of the P_i-dependent phosphorylation of the $(Na^+ + K^+)$-ATPase, both complement the reversal of the over-all reaction sequence observed in red cell preparations (37–39).

The observation that the phospho-enzyme formed in the presence of Ca^{++} is resistant to K^+ and very sensitive to ADP constitutes the first identification of E_1—P in the native, uninhibited $(Na^+ + K^+)$-ATPase. Theoretically it should be possible to demonstrate a sequential relationship between the E_1—P and E_2—P intermediates either by chelating out the Ca^{++} or by adding excess Mg^{++}. However, the effect of adding EGTA and Mg^{++} was only partial and simply reduced the sensitivity of the intermediate to ADP. In other experiments variable changes in the sensitivity of the

Ca^{++}-dependent phospho-enzyme to K^+ after the addition of Mg^{++} and EGTA were observed, but these changes were not statistically significant. This inability of Mg^{++} to overcome the action of Ca^{++} agrees with the noncompetitive interaction between these two ions observed in Fig. 5. Nevertheless, the ability of Na^+ to increase the proportion of the phospho-enzyme which reacted with ADP and the ability of Mg^{++} to reduce its reactivity with ADP is supportive evidence for the postulated E_1—P $\rightleftharpoons$ E_2—P sequence of intermediates in the reaction mechanism of this enzyme (3; but see ref. 1). These observations also agree with the recent observations of Blostein and Burt (8), who concluded that the actions of NEM on the (Na^+ + K^+)-ATPase may not be due entirely to NEM per se but also to Ca^{++}, NEM acting to sensitize the enzyme system to endogenous Ca^{++}.

Similar experiments[2] on the effects of Na^+, Ca^{++}, and ADP on the E_1—P $\rightleftharpoons$ E_2—P equilibrium in guinea pig kidney preparations have shown minimal (though qualitatively similar) effects, suggesting tissue- or species-dependent differences (23, 40). These differences may be related to the physiological role of renal (Na^+ + K^+)-ATPase. Cole and Dirks (41) have shown that renal (Na^+ + K^+)-ATPase is quite insensitive to the inhibitory effects of high concentrations of Na^+. They suggested that this resistance to Na^+ inhibition is related to the action *in vivo* of renal medullary (Na^+ + K^+)-ATPase, which must pump against higher (300 mM) concentrations of Na^+ than the pump enzymes of other tissues. Therefore it seems reasonable that the E_1—P to E_2—P transformation, which energizes Na^+ transport (3), should be less sensitive to high concentrations of Na^+ in enzymes of renal origin.

The observation that Ca^{++} stimulates phosphorylation of the (Na^+ + K^+)-ATPase without stimulating [^{3}H]ouabain binding offers strong support for the hypothesis that the sodium-stimulated binding of [^{3}H]ouabain is related to the sodium-stimulated formation of the E_2—P form of the phospho-enzyme (3, 12, 22). Previous experiments with NEM-treated enzymes showed no ouabain binding to the phospho-enzyme at 0°, although binding occurred at 23° (3). The present results show that the rat brain enzyme can be phosphorylated at 37° in the presence of Ca^{++} without stimulating an equivalent amount of [^{3}H]ouabain binding. For the full binding potential of the phospho-enzyme formed in the presence of Ca^{++}, Mg^{++} must be present, the same condition required for the appearance of K^+ sensitivity. Therefore the results suggest that Na^+E_1ATP and $Na^+Ca^{++}E_1$—P are non-ouabain-binding species, in agreement with the recent observations of Hansen *et al.* (42) and Tobin *et al.* (24) that $Na^+Mg^{++}E_1$ADP is not a binding species. These observations are consistent with schemes which attribute a primary role to the Na^+-stimulated formation of phospho-enzyme in the Na^+-stimulated mechanism of [^{3}H]ouabain binding (3, 12–14, 22, 24).

Recent observations suggest that the interaction of Ca^{++} with the (Na^+ + K^+)-ATPase may have considerable physiological significance. The entry of Ca^{++} into liver cells (43), aplysia neurons (44), and red blood cells (45) increases K^+ exchange by these tissues. In the red cell this effect parallels the depletion of intracellular ATP (46), and since it is inhibited by ouabain, oligomycin, and furosemide, it is considered to proceed via the Na^+ pump mechanism (45). It appears that in the absence of ATP the interaction of Ca^{++} with the enzyme allows the pump to mediate an efflux of K^+ from the cell. This action of Ca^{++}, like its inhibition of (Na^+ + K^+)-ATPase, is intracellular (47, 48). These observations suggest a possible action of Ca^{++} between the formation of K^+E_2 and the binding of ATP to the enzyme, which is apparently a rate-limiting step in the (Na^+ + K^+)-ATPase reaction (33). It appears reasonable that in the presence of low concentrations of ATP Ca^{++} or Ca^{++} plus K^+ could stabilize a particular configuration of the enzyme and that an important action of Ca^{++} in the over-all reaction sequence might be to delay the formation of the Na^+E_1MgATP transphosphorylation complex.

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