The Substitution of Calcium for Magnesium in H⁺,K⁺-ATPase Catalytic Cycle

EVIDENCE FOR TWO ACTIONS OF DIVALENT CATIONS*

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(Received for publication, February 1, 1989)

In order to determine the role of divalent cations in the reaction mechanism of the H⁺,K⁺-ATPase, we have substituted calcium for magnesium, which is required by the H⁺,K⁺-ATPase for phosphorylation from ATP and from P⁰₄. Calcium was chosen over other divalent cations assayed (barium and manganese) because in the absence of magnesium, calcium activated ATP hydrolysis, generated sufficiently high levels of phosphoenzyme (573 ± 51 pmol·mg⁻¹) from [γ-³²P]ATP to study dephosphorylation, and inhibited K⁺-stimulated ATP hydrolysis. The Ca⁺⁺-ATPase activity of the H⁺,K⁺-ATPase was 40% of the basal Mg⁺⁺-ATPase activity. However, the Ca⁺⁺,K⁺-ATPase activity (minus the Ca⁺⁺ basal activity) was only 0.7% of the Mg⁺⁺,K⁺-ATPase, indicating that calcium could partially substitute for Mg⁺⁺ in activating ATP hydrolysis but not in K⁺ stimulation of ATP hydrolysis. Approximately 0.1 mM calcium inhibited 50% of the Mg⁺⁺-ATPase or Mg⁺⁺,K⁺-ATPase activities. Inhibition of Mg⁺⁺,K⁺-ATPase activity was not competitive with respect to K⁺. Inhibition by calcium of Mg⁺⁺,K⁺ activity p-nitrophenyl phosphatase activity was competitive with respect to Mg⁺⁺ with an apparent Ki of 0.27 mM. Proton transport measured by acridine orange uptake was not detected in the presence of Ca⁺⁺ and K⁺. In the presence of Mg⁺⁺ and K⁺, Ca⁺⁺ inhibited proton transport with an apparent affinity similar to the inhibition of the Mg⁺⁺,K⁺-ATPase activity. The site of calcium inhibition was on the exterior of the vesicle. These results suggest that calcium activates basal turnover and inhibits K⁺ stimulation of the H⁺,K⁺-ATPase by binding at a cytosolic divalent cation site.

The pseudo-first-order rate constant for phosphoenzyme formation from 5 μM [γ-³²P]ATP was at least 22 times slower in the presence of calcium (0.015 s⁻¹) than magnesium (0.310 s⁻¹). The Ca⁺⁺-EP (phosphoenzyme formed in the presence of Ca⁺⁺) formed dephosphorylated four to five times more slowly that the Mg⁺⁺-EP (phosphoenzyme formed in the presence of Mg⁺⁺) in the presence of 8 mM trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) or 250 μM ATP. Approximately 10% of the Ca⁺⁺-EP formed was sensitive to a 100 mM KCl chase compared with >85% of the Mg⁺⁺-EP. By comparing the transient kinetics of the phosphoenzyme formed in the presence of magnesium (Mg⁺⁺-EP) and calcium (Ca⁺⁺-EP), we found two actions of divalent cations on dephosphorylation. Dephosphorylation was three times faster with 8 mM CDTA than with 250 μM ATP, regardless of whether the EP was formed with Mg or Ca, suggesting that only one action of the divalent cation, at 2 mM, is inhibition of dephosphorylation and that the divalent cation dissociates rapidly from this site. The persistence of difference in the rates of Mg⁺⁺-EP and Ca⁺⁺-EP dephosphorylation during the CDTA chase indicates that this inhibitory site is different from the divalent cation site required for catalysis. The Ca⁺⁺-EP also dephosphorylated at least 10-20 times more slowly than the Mg⁺⁺-EP in the presence of 10 mM KCl with either 8 mM CDTA or 1 mM ATP. The inability of the Ca⁺⁺-EP to dephosphorylate in the presence of K⁺, compared with the Mg⁺⁺-EP, demonstrates a second action of the divalent cation: that the type of divalent cation which occupies the catalytic divalent cation site required for phosphorylation is important for the conformational transition to a K⁺-sensitive phosphoenzyme and is distinct from the inhibitory divalent cation site previously mentioned. The slower rates of Ca⁺⁺-EP dephosphorylation, compared with the Mg⁺⁺-EP, in the presence of a chelator (with or without KCl), suggests that calcium is tightly bound to the divalent cation site of the phosphoenzyme and the occupation of this site by calcium causes slower phosphoenzyme kinetics.

Phosphorylating transport ATPases bind a variety of ligands during the course of phosphoenzyme intermediate (EP) formation and breakdown. It has been hypothesized that binding of these ligands to phosphorylating ATPases produced changes in conformation that expose cation transport site(s) on the ATPase to the cytosolic (E₁) or extracellular (E₂) surface of the protein. In the case of the H⁺,K⁺-ATPase, transported protons are thought to bind to the E₁ conformation and to be released from the E₁:P conformation. Transported potassium ions are thought to bind to the E₂:P conformation and to be released from the E₁:P conformation (1). The formation of the phosphoenzyme intermediate, E₁:P, from ATP requires Mg⁺⁺ and is accelerated by increasing cytosolic H⁺ concentration, i.e. by the formation of H⁺H⁺ (3). This intermediate can potentially react with ADP to reform ATP or convert to E₁:P. The breakdown of E₁:P is rate-limiting

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1 The abbreviations used are: EP, phosphoenzyme; E₁, cytosolic conformation of the monovalent cation site(s); E₂, extracellular conformation of the monovalent cation site(s); Pipes, piperazine-N,N',N'-bis(2-ethanesulfonic acid); pNPP, p-nitrophenyl phosphate; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; EGTA, ethylenediamine(oxethylenetri)tetraacetic acid; Ca⁺⁺-EP, phosphoenzyme formed in the presence of Ca⁺⁺; Mg⁺⁺-EP, phosphoenzyme formed in the presence of Mg⁺⁺.
in the absence of the K⁺ (3, 4). In the presence of extracytosolic K⁺, E₂P dephosphorylates to form E₁K⁺ at a rate that is faster than enzyme turnover (4). Under these conditions, the rate-limiting step is assumed to occur after dephosphorylation but to precede phosphorylation. The dephosphorylation reaction is thought to require Mg²⁺ because phosphorylation from PO₄ and ^32O-P⁴-O₄ medium exchange require Mg²⁺ (5, 6). The E₂K⁺ form converts to the E₁K⁺ form of the enzyme to release K⁺ into the cytosol, rebind H⁺, and to initiate a new catalytic cycle with the binding of ATP.

The biochemical data accumulated thus far, suggest a similarity in mechanism and structure for the H⁺,K⁺-ATPase and other enzyme phosphorylating ion transport ATPases, such as the Na⁺,K⁺ and sarcoplasmic reticulum Ca²⁺-ATPases (7–9). Four partial reactions are used here to describe the reaction cycle for H⁺,K⁺-ATPase.

Phosphoenzyme formation:

\[ E_1 + ATP \rightleftharpoons E_1P + ADP \]  

(1)

Conformational change from E₁P to E₂P:

\[ H^+ \rightleftharpoons E_2P \]  

(2)

Dephosphorylation:

\[ Mg^{2+} + K^+ \rightleftharpoons E_2P \]  

(3)

Conformational change from E₂ to E₁:

\[ K^+ \rightleftharpoons E_1 \]  

(4)

These four reactions are common to EP ion translocating ATPases and have been elaborated for the Na⁺,K⁺ and sarcoplasmic reticulum Ca²⁺-ATPases to account for additional partial reactions such as ion occlusion and passive ion fluxes (10, 11). The conformational changes E₁P to E₂P, and E₁ to E₂ are thought to be associated with the movement of ions from one side of the enzyme to the other.

This paper will focus on the role of the divalent cation in the above partial reactions. The H⁺,K⁺-ATPase requires Mg²⁺ for phosphorylation from ATP and for phosphorylation from PO₄, but there is no direct evidence that Mg²⁺ is required for conformational changes (3–5). To determine the Mg²⁺ requirement in the partial reactions of ATP hydrolysis, Mg²⁺ has been replaced with other divalent cations. Three criteria were used to select a probe of the H⁺,K⁺-ATPase divalent cation site. 1) The divalent cation should substitute for Mg²⁺ for phosphorylation from ATP and for phosphorylation from PO₄ but there is no direct evidence that Mg²⁺ is required for conformational changes (3–5). To determine the Mg²⁺ requirement in the partial reactions of ATP hydrolysis, Mg²⁺ has been replaced with other divalent cations. 3) The level of EP in the presence of the divalent cation should be comparable to the level of EP with Mg²⁺. 3) The divalent cation should not substitute for Mg²⁺ during K⁺-stimulated enzyme turnover. In preliminary experiments Ba²⁺, Mn²⁺, and Ca²⁺ substituted for Mg²⁺ in the formation of phosphoryoenzyme (30, 90, and 60% of the Mg level were, respectively) but only Ca²⁺ yielded high levels of EP and did not substitute for Mg²⁺ in K⁺-stimulated dephosphorylation.

In this report we describe the effect of replacing Mg²⁺ with Ca²⁺ as the divalent cation in the H⁺,K⁺-ATPase reaction cycle. We propose that Ca²⁺ has two types of effects on the H⁺,K⁺-ATPase. 1) Ca²⁺ activates ATP hydrolysis and substitutes for Mg²⁺ in the formation and dephosphorylation of the phosphoryoenzyme with slower kinetics. 2) Ca²⁺ inhibits Mg²⁺-dependent hydrolysis, K⁺-stimulated hydrolysis of ATP or pNP, and proton transport, and does not substitute for Mg²⁺ in the K⁺-stimulated dephosphorylation reaction of EP.

**EXPERIMENTAL PROCEDURES**

**Reagents**

[^7-32P]ATP was purchased from Amersham Corp. and "vanadium free" disodium ATP was obtained from Sigma. All other reagents were at least of reagent grade.

**Methods**

**Enzyme Preparation**—The porcine gastric H⁺,K⁺-ATPase was prepared according to previously published methods (12). Briefly, scrapings from stomachs obtained at slaughter were homogenized in 0.25 m sucrose buffered with 4 mM Pipes-Tris, pH 7.4. The homogenate was centrifuged at 20,000 x g for 45 min with a Sorvall GSA rotor. The supernatant from the 20,000 x g spin was then spun at 100,000 x g for 1 h with a Beckman Ti-70 rotor. Pellets were resuspended in 1 ml protein and placed on a 0.25 m sucrose; 0.25 m sucrose, 7% Ficoll solution interface (GL1) and stored at –80 °C. The GL1 material is considered to be freely permeable to ions since the ATPase activity is neither vallinomycin or nigericin stimulated and proton transport was not detected after lyophilization.

**ATPase Assays**—ATPase activity was measured at 37 °C with 5–10 µg of enzyme, and the PO₄ liberated was determined by the method of Yoda and Hokin (13). Final reaction concentrations in 1 ml reaction volume were 2 mM Na₃ATP, 20 mM Tris-C1, pH 7.4 or 7.2, 20 mM KCl and 2 mM MgCl₂, unless otherwise indicated. CaCl₂ concentrations varied from 25 µM to 2 mM. In solutions with no added Ca²⁺, Ca²⁺ was measured by atomic absorption, and the Ca²⁺ concentration was 3–6 µM.

**pNPase Assays**—pNPase activity was measured at 37 °C with 20 µg of enzyme and the p-nitrophenol liberated was measured spectrophotometrically at 405 nm at a pH greater than 10. Final reaction concentrations in 1 ml were 6 mM pNP, 20 mM Tris-C1, pH 7.2, 40 mM KCl and 6 mM MgCl₂, unless otherwise indicated.

**Phosphoenzyme Studies**—Phosphoenzyme experiments were typically carried out with 20–40 µg of protein in a 1-ml reaction volume (30, 90 °C). Final reaction concentrations in the standard reaction solution were 2 mM divalent cation chloride, 40 mM Tris-C1, pH 7.4, 0.3 mM CDTA, and 5 µM[^7-32P]ATP (with a specific activity of 50–200 cpmp⁻μM⁻¹). Phosphoenzyme formation was initiated with the addition of 100 µM of labeled ATP to a 90 or 400 µl (for dephosphorylation experiments) volume of protein preincubated with 10 mM of ATP for 10 min and then placed on ice for at least 10 min. In the dephosphorylation studies, ligands were added in a 100-µl volume to a 900-µl volume of protein preincubated with the labeled ATP for 15 or 180 s for Mg²⁺ and Ca²⁺, respectively. Reactions were stopped with the addition of a 500-µl ice-cold solution of 40% (w/v) trichloroacetic acid, 5 mM H₃PO₄, and 5 µM unlabeled ATP. Trichloroacetic acid-precipitated proteins were filtered within 15 min of reaction termination using a 3 or 0.45 µM HAWP millipore filter. The filter support and test tube were washed four times with 5 ml of an ice-cold solution of 3% trichloroacetic acid and 5 mM H₂PO₄. Filter papers were dried, dispensed into 10 ml of Amersham Corp. counting scintillate and counted using a LKB liquid scintillation counter.

Non specific labeling was measured as the amount of radioactive label incorporated in the presence of 4 mM CDTA, 4 mM EGTA, and 1 mM KCl. Incorporation was usually between 5–10% of the total phosphoenzyme generated with Mg²⁺ and was subtracted as a blank from the total Mg²⁺ or Ca²⁺ formed.

**Transport Assays**—Proton transport was measured using the weak base acridine orange as previously described (14). The pH-dependent absorbance shift at 492 nm was monitored with an Aminco DW-2 spectrophotometer at 22–24 °C. Vesicles were equilibrated at 4°C with the appropriate ions for a minimum of 30 h. Final concentrations of the ions in the transport reaction were the same as in the above partial reactions. The H⁺,K⁺-ATPase requires Mg²⁺ in the formation of phosphoenzyme.
internal vesicular concentrations of ions are indicated in the figure legends.

**Protein Determination**—Protein was measured by the method of Lowry et al. (15) using bovine serum albumin standards.

**Calculations**—The rate constants were calculated by a computer program provided by the Biomathematics unit of CURE. A single exponential provided a reasonably good fit of the data and the standard errors for the rate constants are given in Table II. The data points and associated bars indicate the mean and standard error of two to six experiments. The data points in the figures are connected by an interpolation between the points.

Free Ca$^{2+}$ concentrations were calculated, using values of Martell and Smith (16) for ligand dissociation constants, with a computer program kindly provided by Dr. D. D. F. Loo.

**RESULTS**

**Ca$^{2+}$ Inhibition and Activation of ATPase and pNPPase Activities**

The gastric H$^+$,K$^+$-ATPase exhibits a basal activity in the presence of Mg$^{2+}$ and in the absence of monovalent cations (Mg$^{2+}$-ATPase). At 2 mM Mg$^{2+}$, optimal for the Mg$^{2+}$-ATPase, the inhibitory effect of calcium was examined. Ca$^{2+}$ inhibits the Mg$^{2+}$-ATPase activity (Ca$^{2+}$-ATPase activity was subtracted) with an IC$\textsubscript{50}$ of approximately 100 $\mu$M (Fig. 1).

In the presence of K$^+$ and Mg$^{2+}$, the gastric H$^+$,K$^+$-ATPase demonstrates a stimulated activity (Mg$^{2+}$,K$^+$-ATPase). Calcium completely inhibits the Mg$^{2+}$,K$^+$-ATPase activity with (Ca$^{2+}$,Mg$^{2+}$-ATPase activity was subtracted) an IC$\textsubscript{50}$ for Ca$^{2+}$ of approximately 100 $\mu$M. Presumably Ca$^{2+}$ could prevent monovalent cation stimulation by competitively displacing K$^+$ from an activating site on the enzyme. The simplest expectation of such an interaction by Ca$^{2+}$, that is the apparent $K_i$ for Ca$^{2+}$ would increase as the K$^+$ concentration increased. This was not observed; the IC$\textsubscript{50}$ for Ca$^{2+}$ does not increase significantly over a 40-fold K$^+$ concentration range extending from 0.5 to 20 mM (data not shown). Calcium also inhibited K$^+$ stimulation of the Mg$^{2+}$,K$^+$-pNPPase activity in a competitive manner with respect to Mg$^{2+}$ with an apparent $K_i$ of 0.27 mM (Fig. 1B).

In the absence of Mg$^{2+}$, with only Ca$^{2+}$ present, there was no detectable K$^+$ stimulation of either ATP or pNPP hydrolysis (Table I).

In contrast to the situation where Ca$^{2+}$ inhibits the Mg$^{2+}$-activated enzyme, Ca$^{2+}$ alone activates ATP hydrolysis (Ca$^{2+}$-ATPase) with an activity equaling 40% of the Mg$^{2+}$-ATPase activity (Table I). Half-maximal activation of ATPase activity occurs between 0.4–0.7 mM CaCl$\textsubscript{2}$. The Ca$^{2+}$-ATPase and Mg$^{2+}$-ATPase activities are also inhibited by 45 and 75%, respectively, by 60 $\mu$M SCH 28080, a specific inhibitor of the H$^+$,K$^+$-ATPase (17).

**EP Studies: Inhibition of the Partial Reactions**

**Calcium Slows Phosphoenzyme Formation**—The steady state hydrolysis measurements indicate that calcium can both activate and inhibit H$^+$,K$^+$-ATPase turnover. Kinetic analysis of the partial reactions of the gastric ATPase's phosphoenzyme further defines calcium inhibition and activation of hydrolysis. In the first step, the formation of phosphoenzyme:

$$E + Mg^{2+} + ATP \rightarrow ADP + Mg\cdot EP$$

calcium forms phosphoenzyme from 5 $\mu$M ATP 22 times slower than Mg$^{2+}$ (Fig. 2 and Table II).

In the presence of 0.3 mM CDTA and in the absence of divalent cation, no phosphoenzyme formation occurs, indicating that either Ca$^{2+}$ or Mg$^{2+}$ are necessary to generate Ca$^{2+}$ and Mg$^{2+}$ phosphoenzyme (Ca-EP or Mg-EP, respectively).

**Table I**

| Ligands | Velocity | Ligands | Velocity |
|---------|----------|---------|----------|
| Mg$^{2+}$ | 9 ± 3 (n = 3) | Mg$^{2+}$ | 1.8 ± 0.2 (n = 3) |
| Mg$^{2+}$,K$^+$ | 150 ± 10 (n = 3) | Mg$^{2+}$,K$^+$ | 62 (n = 1) |
| Mg$^{2+}$,Ca$^{2+}$ | 4 ± 1 (n = 3) | Mg$^{2+}$,Ca$^{2+}$ | 1.0 ± 0.2 (n = 3) |
| Mg$^{2+}$,Ca$^{2+}$,K$^+$ | 0 (n = 3) | Mg$^{2+}$,Ca$^{2+}$,K$^+$ | 0 (n = 3) |
| Ca$^{2+}$ | 4 ± 1 (n = 3) | Ca$^{2+}$ | 1.0 ± 0.3 (n = 3) |
| Ca$^{2+}$,K$^+$ | 1 ± 1 (n = 3) | Ca$^{2+}$,K$^+$ | 0.7 ± 1 (n = 3) |

**Velocities are expressed as $\mu$mol of product-mg$^{-1}$-h$^{-1}$ at 37°C.**

The Mg$^{2+}$, K$^+$ or Ca$^{2+}$,K$^+$ activity is the activity of the H$^+$,K$^+$-ATPase in the presence of Mg$^{2+}$ or Ca$^{2+}$ or K$^+$ minus the activity in the presence of Mg$^{2+}$ or Ca$^{2+}$ or K$^+$ alone. The Mg$^{2+}$ and K$^+$ concentration in the ATPase assay are 2 and 20 mM, respectively. B, Ca$^{2+}$ is a competitive inhibitor of Mg$^{2+}$ in the K$^+$-stimulated pNPPase reaction. Calcium concentrations are 6 mM (C), 100 $\mu$M (BR), 300 $\mu$M (E), and 1000 $\mu$M (F), respectively. Assay conditions are discussed under "Experimental Procedures" and unnormalized activities are found in Table I.
The slower phosphorylation kinetics induced by Ca\textsuperscript{2+} was also observed by Tobin et al. (18) for the Na\textsuperscript{+},K\textsuperscript{+}-ATPase. Slower phosphorylation of the H\textsuperscript{+},K\textsuperscript{+}-ATPase \((k_1 = 0.011 \text{ s}^{-1} \pm 0.001)\) in the presence of calcium was seen at 0.5 \(\mu\text{M}\) ATP as well (19).

Maximal levels of phosphoenzyme formed on ice were 970 \pm 68 pmol-mg\textsuperscript{-1} of protein for the Mg\textsuperscript{2+} EP (n = 17) and 573 \pm 51 pmol-mg\textsuperscript{-1} of protein for the Ca\textsuperscript{2+} EP \((n = 22)\). The Mg\textsuperscript{2+} EP level agrees well with results published by Wallmark and Mårdh at 22 °C (3). The ratio of Ca\textsuperscript{2+}-EP/Mg\textsuperscript{2+}-EP of the gastric H\textsuperscript{+},K\textsuperscript{+}-ATPase is nearly identical to the Ca\textsuperscript{2+}/Mg\textsuperscript{2+} EP ratio found by Fukushima and Post (20) and Tobin et al. (18) for the Na\textsuperscript{+},K\textsuperscript{+}-ATPase.

The phosphorylated intermediate generated in the presence of Ca\textsuperscript{2+} exhibits the same chemical characteristics as the Mg\textsuperscript{2+} generated EP. Both types of EP are base labile, acid stable, and 85% hydroxylamine sensitive (21).

**Calcium Slows Dephosphorylation**—In the presence of Mg\textsuperscript{2+} alone the phosphoenzyme spontaneously reacts with water

\[
\text{Mg-EP} + \text{H}_2\text{O} \rightarrow \text{E} + \text{P} + \text{Mg}^{2+}
\]

This reaction is slow relative to that found when K\textsuperscript{+} is included with Mg\textsuperscript{2+} in the reaction media (4). Dephosphorylation is measured after abolishing phosphorylation in one of two ways: 1) by diluting the labeled \([\gamma^{32P}]\text{ATP}\) 50-200-fold with the addition of unlabeled ATP or, 2) by the addition of a chelator in excess of the divalent cation concentration. Fig. 3, A and B, shows that the replacement of magnesium with calcium slows another partial reaction of the phosphoenzyme. In this case, dephosphorylation of the Ca\textsuperscript{2+} EP is four to five times slower than that of the Mg\textsuperscript{2+} EP, by either method of determining dephosphorylation. Comparison of the chelator and unlabeled ATP\textsuperscript{2-} induced dephosphorylation rates for either the Ca\textsuperscript{2+} or Mg\textsuperscript{2+}, EPs assesses the effect of free divalent cation on the dephosphorylation rate (Fig. 3, A and B). Apparently, chelator-dependent breakdown of either the Mg\textsuperscript{2+} or Ca\textsuperscript{2+} EP is faster than their corresponding unlabeled ATP-dependent breakdown (Table II), suggesting that chelator accessible divalent cation inhibits the dephosphorylation reaction. The possibility that the slower unlabeled ATP-induced dephosphorylation rates were due to ATP inhibition of dephosphorylation is unlikely since the dephosphorylation rate did not vary as a function of concentration of unlabeled ATP added (25-1000 \(\mu\text{M}\), providing there was sufficient \(250 \mu\text{M}\) unlabeled ATP to observe dephosphorylation (data not shown). The lack of inhibition by ATP was also shown by Ray and Forre (22).

**Ca\textsuperscript{2+} EP Is ADP Insensitive**—Since calcium slows phosphorylation, inhibits K\textsuperscript{+} stimulation of ATPase activity, and maintains a K\textsuperscript{+}-insensitive EP (as discussed in the following section) an expected correlate would be the generation of an ADP sensitive Ca\textsuperscript{2+} EP. This hypothesis was tested by forming EP in the presence of Ca\textsuperscript{2+} and subsequently adding ATP. Since ADP addition could lead to EP dephosphorylation via two mechanisms: 1) by prevention \([\gamma^{32P}]\text{ATP}\) from forming EP or, 2) by reacting with previously formed EP, the ADP induced dephosphorylation was compared with the unlabeled ATP dephosphorylation which measures the former mechanism. The Ca\textsuperscript{2+} EP did not dephosphorylate faster with ADP than with ATP, like the ADP-insensitive phosphoenzyme found by Rabon et al. (23) (Table II).

**Ca\textsuperscript{2+} EP Is K\textsuperscript{+} Insensitive**—When K\textsuperscript{+} is added to the Mg\textsuperscript{2+} EP there is a rapid phase of dephosphorylation as a new

![Fig. 2. Ca\textsuperscript{2+} substitutes for Mg\textsuperscript{2+} in the formation of phosphoenzyme with slower kinetics. At time zero the reaction was started with the addition of \([\gamma^{32P}]\text{ATP}\) at the indicated times and was stopped with 40% trichloroacetic acid, 5 mM H\textsubscript{3}PO\textsubscript{4}, and 5 mM ATP. Reactions are on ice. With 5 \(\mu\text{M}\) ATP and 2 mM MgCl\textsubscript{2} or CaCl\textsubscript{2} the maximal amount of Mg\textsuperscript{2+}-EP formed is 970 pmol-mg\textsuperscript{-1} \pm 68 \((n = 17)\) and the maximal amount of Ca\textsuperscript{2+}-EP formed is 573 pmol-mg\textsuperscript{-1} \pm 51 \((n = 22)\) (3). The ratio of Ca\textsuperscript{2+}-EP/Mg\textsuperscript{2+}-EP equals 0.6. Assay conditions are discussed under "Experimental Procedures" and the pseudo-first-order rate constants are found in Table II.](image-url)
Fig. 3. Dephosphorylation is faster in the presence of a chelator for both Mg-EP and Ca-EP. At time zero, dephosphorylation is initiated with the addition of a 20-fold excess of ATP (○) or 8 mM chelator (CDTA or EGTA) (○) and at the indicated times stopped with 40% trichloroacetic acid, 5 mM H$_3$PO$_4$, and 5 mM ATP. For Mg-EP time zero is 15 s after the addition of [γ-32P]ATP, formed as in Fig. 2. For Ca-EP time zero is 180 s after the addition of [γ-32P]ATP, formed as in Fig. 2. In A the rate for Mg-EP dephosphorylation initiated with a chelator is compared with the rate initiated with excess ATP. In B the rate for Ca-EP dephosphorylation initiated with a chelator is compared with the rate initiated with excess ATP. Reactions are on ice. Assay conditions are discussed under "Experimental Procedures." The first order rate constants are found in Table I.

steady state level of EP is approached (3, 4). Ca$^{2+}$ prevents the initial rapid phase of K$^+$-stimulated dephosphorylation of EP (Fig. 4A). Even after 120 s when a new steady state level of EP is reached only 10% of the Ca-EP is labile to 0.1 mM KCl while 65% of the Mg-EP is K$^+$ labile. Abolition of the rapid phase of K$^+$-induced dephosphorylation of EP is more pronounced at 100 mM KCl. After 10 s only 15% of the Mg-EP remains while over 90% of the Ca-EP is stable to these conditions (Fig. 4B).

K$^+$ stimulation of phosphoenzyme breakdown was also measured when phosphorylation was prevented by the addition of unlabeled ATP as shown in Fig. 5A. Under these conditions the Ca-EP dephosphorylates 10–20 times more slowly than the Mg-EP (Table II). K$^+$ increases the Ca-EP rate of dephosphorylation measured in the presence of unlabeled ATP only 3-fold. This is in contrast to the Mg-EP where K$^+$ increases the dephosphorylation rate in the presence of unlabeled ATP greater than 20-fold.

To assess the effect of free divalent cation on K$^+$ stimulation of EP dephosphorylation, phosphorylation from ATP was prevented by the removal of divalent cation using a chelator (Fig. 5B). Under these conditions the Ca-EP dephosphorylates at least 10–20 times more slowly than the Mg-EP (Table II). The rate constant of dephosphorylation of the Ca-EP in the presence of a chelator is increased 3-fold by K$^+$. In contrast, K$^+$ increases the rate constant for dephosphorylation of Mg-EP in the presence of a chelator at least 20-fold. Apparently Ca$^{2+}$ blocks K$^+$ stimulation of dephosphorylation of EP even after chelation, suggesting either that Ca$^{2+}$ dissociated from the phosphoenzyme, conferring slower K$^+$-stimulated dephosphorylation kinetics or that Ca$^{2+}$ tightly bound to the enzyme is responsible for the slower kinetics.

Inhibition of Proton Transport

The Sideness of Ca Inhibition—In the hydrolysis and phosphoenzyme experiments lyophilized vesicles were used, with access of Ca$^{2+}$ to both the cytosolic and extracellular face of the enzyme. The predominantly inside-out orientation of the gastric G1 vesicle fraction was exploited to assess whether the Ca$^{2+}$ inhibitory site is located on the lumenal or cytosolic face of the H$^+$,K$^+$-ATPase. The data described thus far points toward a cytosolic site of inhibition. The acridine orange uptake data in Fig. 6A show that external Ca$^{2+}$ does not activate proton transport and that internal Ca$^{2+}$ does not
Catalysis of H⁺,K⁺-ATPase Divalent Cation Site

FIG. 5. K⁺-stimulated dephosphorylation is blocked in Ca⁺-EP, compared with Mg⁺-EP, even in the presence of a chelator. In A the Ca⁺-EP (O) dephosphorylates in the presence of K⁺ and ATP much slower than the Mg⁺-EP (●). In B the Ca⁺-EP (O) dephosphorylates in the presence of K⁺ and a chelator much slower than the Mg⁺-EP (●). At time zero dephosphorylation is initiated with the addition of 10 mM KCl and 1 mM ATP (A) or 10 mM KCl and 8 mM CDTA or EGTA (B) and, at the indicated times, stopped with 4% trichloroacetic acid, 5 mM H₂PO₄, and 5 mM ATP. For Mg-EP time zero is 15 s after the addition of 5 μM [γ³²P]ATP. For Ca-EP time zero is 180 s after the addition of 5 μM [γ³²P]ATP. Assay conditions are discussed under “Experimental Procedures” and the first order rate constants are found in Table II.

Inhibit proton transport. Control vesicles equilibrated with 150 mM KCl and diluted into 150 mM KCl and 1 mM MgCl₂ show a rapid uptake of the lipophilic weak base acridine orange as a consequence of ATP addition. The change in absorbance indicates that protons and acridine orange were transported and trapped inside the vesicle lumen (recording A). Vesicles equilibrated with 150 mM KCl and 1 mM CaCl₂ and diluted into 150 mM KCl and 1 mM MgCl₂ displayed an acridine orange uptake response to the addition of ATP nearly identical to that of the control (recording B). The slightly slower rate of acridine orange uptake into the CaCl₂ equilibrated vesicles is explained by external Ca²⁺ present from diluting the equilibrated vesicles into the assay solution. Vesicles with both internal and external KCl and 1 mM external CaCl₂ showed no proton transport. To ensure that CaCl₂ was in the lumen of the vesicles, vesicles equilibrated with CaCl₂ and KCl were diluted into a KCl assay solution. Addition of the Ca²⁺/H⁺ exchanger A23187 caused proton uptake into the vesicles via the outwardly directed CaCl₂ gradient produced upon dilution of the vesicles into the assay solution. Fig. 6B shows that external Ca²⁺ inhibits proton transport with an IC₅₀ similar to that observed for inhibition of the Mg⁺⁺- and Mg⁺⁺,K⁺-ATPase activities. The requirement for external Ca²⁺ for inhibition of proton transport indicates that the Ca²⁺ inhibitory site is on the cytosolic face of the enzyme.

DISCUSSION

The present investigation demonstrates the activating and inhibitory effects of Ca²⁺ on H⁺,K⁺-ATPase turnover and partial reactions of the ATPase.

Activation—The Ca²⁺-ATPase activity, the Ca²⁺-pNPPase activity, and the formation and dephosphorylation of Ca⁺-EP demonstrate that Ca²⁺ activates the H⁺,K⁺-ATPase (Figs. 2 and 3 and Table I). The activation of the H⁺,K⁺-ATPase by

FIG. 6. Ca²⁺ inhibition site is cytosolic, determined using proton transport in inside-out vesicles. A shows trace A: control for Mg-ATP dependent proton transport measured by acridine orange uptake into vesicles equilibrated with 150 mM external KCl and diluted into 150 mM KCl and 1 mM MgCl₂; trace B: internal Ca²⁺ does not inhibit proton transport; trace C: proton transport is not supported by external Ca²⁺; trace D: control for CaCl₂ equilibration of vesicles; the addition of the Ca²⁺/H⁺ exchanger A23187 promotes formation of a proton gradient. B shows the inhibition by 0, 0.015, 0.075, 0.125, 0.250, 0.500 mM external Ca²⁺ on Mg-ATP-dependent proton transport. Assay conditions are discussed under “Experimental Procedures.” The K⁺/H⁺ exchanger nigericin is abbreviated using NIG. Addition of nigericin reversed the signals indicating a proton gradient was present in the vesicles.
Ca"^2+ is explained by Ca"^2+ substituting for Mg"^2+ as a divalent cation in the catalytic cycle in the absence of K"^+.

Inhibition of K"+-stimulated Hydrolysis and Transport—When Ca"^2+ replaces Mg"^2+ as the activating divalent cation, K"^+-stimulation of the catalytic cycle is less. The rapid phase of K"^+ dephosphorylation of the Mg-EP does not occur with the Ca-EP and K"^+ is unable to accelerate greatly the breakdown of the Ca-EP (Figs. 4 and 5). In the presence of Ca"^2+ and the absence of Mg"^2+, all K"^+ stimulation of ATP and pNPP hydrolysis is inhibited and only the divalent cation catalyzed turnover is present.

Calcium Inhibition Site—Recently, it has been shown for the Na"^+,K"^+-ATPase that calcium binds to a K"^+ site on the enzyme and does so probably through an E1 conformation (24, 25). These observations raise the question as to whether Ca"^2+ blocks K"+-stimulated enzyme turnover and dephosphorylation by binding at a K"^+ site or a Mg"^2+ site on the H"^+,K"^+-ATPase. Ca"^2+ inhibition of K"^+ stimulation at the monovalent cation activation site on the H"^+,K"^+-ATPase is unlikely for the following reasons: 1) Ca"^2+ inhibition of K"+-stimulated hydrolysis is competitive with Mg"^2+ and not K"^+ (Fig. 1), 2) the K"^+ activation site is luminal and the Ca"^2+ inhibition site is cytosolic (Fig. 6, A and B), 3) high concentrations of K"^+ in the presence of CDTA increase the rate constant for dephosphorylation of the Ca-EP only slightly compared with the effect of K"^+ on Mg-EP (Fig. 4, A and B), 4) in contrast to the Na"^+,K"^+-ATPase, Ca"^2+ slows the kinetics of the partial reactions and has no K"^+ like effect on the phosphoenzyme (24).

The evidence for Ca"^2+ replacing Mg"^2+ at the divalent cation site is the following: Ca"^2+ substitutes for Mg"^2+ in activating the H"^+,K"^+-ATPase, inhibits the Mg"^2+-ATPase, is a competitive inhibitor with respect to Mg"^2+ of the Mg"^2+,K"^+-ATPase, has a cytosolic site of action and forms an EP in the absence of Mg"^2+ (Table I, Figs. 1, A and B, and 2, A and B). These data support the conclusion that calcium replaces magnesium. Alternatively, it could be argued that contaminating Mg"^2+ bound to H"^+,K"^+-ATPase is not exchanged for Ca"^2+, because it has a much higher affinity for the enzyme than Ca"^2+ and that Ca"^2+ inhibition must be due to Ca"^2+ binding at a second divalent cation site that is inhibitory. If that is the case, then it follows that removal or chelation of the Ca"^2+ dissociating from the inhibitory site on the Ca-EP by either CDTA or EGTA would restore Mg-EP dephosphorylation kinetics to the phosphoenzyme. This is not the case. Chelation of Ca"^2+ by either CDTA or EGTA does not confer the faster Mg-EP type kinetics of dephosphorylation to the Ca-EP (Fig. 5B). Furthermore Mg"^2+ bound to the H"^+,K"^+-ATPase, from μM Mg"^2+ contamination, must be exchangeable with the 1–2 mM Ca"^2+ in the assay solutions, since chelation of divalent cation blocks phosphoenzyme formation (unless the magnesium affinity for the enzyme is 1000 times greater than calcium). Evidently, Ca"^2+ can replace the Mg"^2+ bound to the enzyme rather than modify a Mg-EP at a second, inhibitory site.

Ca"^2+ Cannot Substitute for Mg"^2+ in K"+-stimulated Dephosphorylation of EP—Both the Ca-EP and the Mg-EP dephosphorylate faster in the presence of a chelator compared with unlabeled ATP and are ADP insensitive. However, only the Mg-EP is predominantly K"^+ sensitive since only 5–10% of the Ca-EP is sensitive to 0.1 mM KCl compared with 55–60% of the Mg-EP (Fig. 4A). The rate constant for EP dephosphorylation in the presence of a chelator or unlabeled ATP is 20–30 times faster with K"^+ for Mg-EP but only three times faster for the Ca-EP. These data indicate that Mg"^2+ is required for Mg-EP K"^+-dephosphorylation.

Evidence for Two Types of Divalent Cation Sites—Dephosphorylation is faster in the presence of a chelator than in the presence of unlabeled ATP in the absence or presence of K"^+ for both the Ca-EP and the Mg-EP (Figs. 3, A and B and 5, A and B). The differences in rates suggest that there is an inhibitory divalent cation site, which slows dephosphorylation regardless of the type of divalent cation used for phosphorylation and that the divalent cation can rapidly dissociate from this site. The inhibitory site is different from the site required for catalysis since differences in the dephosphorylation kinetics of the Mg-EP and the Ca-EP persist after removal of free divalent cation. This inhibitory site maybe due to the interaction of the divalent cation at the monovalent cation site required for dephosphorylation.

The other divalent cation site is required for phosphoenzyme formation and possibly dephosphorylation. Calcium can partially substitute for magnesium in the catalytic divalent cation site when catalysis by the H"^+,K"^+-ATPase is K"^+ independent (Table I). However, the kinetics of K"^+-independent hydrolysis, phosphoenzyme formation, and dephosphorylation are slower with calcium than with magnesium (Figs. 2, 3, A and B, Table I), suggesting that the substitution of calcium for magnesium is incomplete and that catalysis of K"^+-independent turnover by the divalent cation is selective for magnesium over calcium. It is possible that the replacement of magnesium by calcium completely alters the enzyme’s catalytic mechanism, leaving the enzyme incapable of undergoing conformational changes between E,P and E,EP. However the Mg"^2+ and Ca"^2+ EP share properties of being ADP insensitive, are inhibited by divalent cations and are comparable in steady state levels. Furthermore, the differences in rate constants of the K"^-independent partial reactions do not warrant a different catalytic mechanism for K"^-independent turnover.

The small proportion of E,EP and slow K"+-stimulated dephosphorylation kinetics of the Ca-EP (Figs. 4, A and B, and 5, A and B), compared with the Mg-EP, suggests that the catalytic divalent cation site requires occupation by Mg"^2+, rather than Ca"^2+, for conformational changes and K"^+ stimulation of dephosphorylation, as well as for normal rates of K"^-independent catalysis. If divalent cation is required for catalysis, it may be that calcium dissociates more rapidly from EP than magnesium and only the Mg-EP complex is stable enough to permit rapid dephosphorylation.

Our experiments do not support the conclusion that the dissociation rate of the divalent cation from the catalytic site determines the rate of K"+-stimulated dephosphorylation. In experiments where the divalent concentration was near saturating (1–2 mM), and divalent cation should be bound to the EP, the rates of dephosphorylation with ATP of the Mg-EP and the Ca-EP are different in the absence of K"^+, kₚ = 0.014 s⁻¹ and k₋ = 0.003 s⁻¹, respectively, and in the presence of K"^+, kₚ = 0.22 s⁻¹ and k₋ = 0.014 s⁻¹, respectively (Figs. 3, A and B, and 5A). Hence, the type of divalent cation occupying the catalytic divalent cation site is responsible for the difference in dephosphorylation kinetics. The difference in rates of dephosphorylation of the Mg-EP and the Ca-EP are maintained after chelation; in the absence of K"^+, kₚ = 0.038 s⁻¹ and k₋ = 0.009 s⁻¹, respectively, and in the presence of K"^+, kₚ > 0.35 s⁻¹ and k₋ = 0.029 s⁻¹. Since the difference in dephosphorylation rates between Mg-EP and Ca-EP is dependent on the type of divalent cation used for the formation of phosphoenzyme and not the presence of free divalent cation in solution, it follows that neither magnesium nor calcium have dissociated from the divalent cation site and that the
divalent cation, that remains bound to the phosphoenzyme is responsible for the differences in transient kinetics.

Acknowledgments—We thank Iqbal Anwar for his expert assistance with the pNPPase assays, David Scott for his expert photography, Azita Ashofteh, Donna Tomlinson, and Velma Seiwan for their help in preparing the manuscript.

REFERENCES
1. Sachs, G., Kaunitz, J., Mendlein, J., and Wallmark, B. (1989) The Handbook of Physiology, in press
2. Stewart, B., Wallmark, B., and Sachs, G. (1981) J. Biol. Chem. 256, 2682-2690
3. Wallmark, B., and Márđh, S. (1979) J. Biol. Chem. 254, 11899-11902
4. Wallmark, B., Stewart, H. B., Rabon, E., Saccomani, G., and Sachs, G. (1980) J. Biol. Chem. 255, 5313-5319
5. Jackson, R. J., and Saccomani, G. (1984) Biophys. J. 45, 839a
6. Faller, L. D., and Elgavish, G. A. (1984) Biochemistry 23, 6584-6590
7. Shull, G. E., Schwartz, A., and Lingrel, J. B. (1985) Nature 316, 691-695
8. Maclennan, D. H., Brandl, C. J., Korczak, B., and Green, N. M. (1985) Nature 316, 696-700
9. Shull, G. E., and Lingrel, J. B. (1986) J. Biol. Chem. 261, 16788-16791
10. Karlish, S. J. D., Lieb, W. R., and Stein, W. D. (1982) J. Physiol. 328, 33-350
11. Wakabayashi, S., and Shigekawa, M. (1987) J. Biol. Chem. 262, 11524-11531
12. Sachs, G., Chang, H. H., Rabon, E., Schackman, R., Lewin, M., and Saccomani, G. (1976) J. Biol. Chem. 251, 7690-7698
13. Yoda, A., and Hokin, L. E. (1970) Biochem. Biophys. Res. Commun. 40, 880-886
14. Rabon, E., Chang, M., and Sachs, G. (1978) Biochemistry 17, 3345-3353
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
16. Martell, A. E., and Smith, R. M. (1974) Critical Stability Constants. Volume 1, Amino Acids, Plenum Press, New York
17. Wallmark, B., Briving, C., Fryklund, J., Munson, K., Jackson, R., Mendlein, J., Rabon, E., and Sachs, G. (1987) J. Biol. Chem. 262, 2077-2084
18. Tobin, T., Akera, T., and Brody, T. M. (1975) Biochim. Biophys. Acta 389, 117-125
19. Jackson, R. J., Mendlein, J., and Sachs, G. (1983) Biochim. Biophys. Acta 731, 9-15
20. Fukushima, Y., and Post, R. L. (1978) J. Biol. Chem. 253, 6853-6862
21. Caroni, P., and Carafoli, E. (1981) J. Biol. Chem. 256, 9371-9373
22. Ray, T. K., and Forte, J. G. (1976) Biochim. Biophys. Acta 443, 451-457
23. Rabon, E., Sachs, E., Márđh, S., and Wallmark, B. (1982) Biochim. Biophys. Acta 688, 515-524
24. Vasallo, P. M., and Post, R. (1986) J. Biol. Chem. 261, 16957-16962
25. Forbush, B., Jones, G., and Barberia, J. T. (1986) J. Gen. Physiol. 88, 23a-24a