Comparison between Calcium Transport and Adenosine Triphosphatase Activity in Membrane Vesicles Derived from Rabbit Kidney Proximal Tubules*

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Characteristics of Ca\(^{2+}\) uptake were studied in a vesicular preparation of proximal tubule plasma membranes from rabbit kidney and compared with the properties of both membrane-bound and solubilized Ca\(^{2+}\)-ATPase activities. Calcium uptake required both ATP and MgCl\(_2\) and revealed two kinetic components with respect to Ca\(^{2+}\) concentration requirements, one with a high affinity for Ca\(^{2+}\) (1.8 \(\mu\)M), operative in the range of cytosolic Ca\(^{2+}\) activity, and one with a low affinity for Ca\(^{2+}\) (250 \(\mu\)M) which may become active only at abnormally high cytosolic Ca\(^{2+}\) concentrations. The high- and low-affinity components were stimulated to similar extents by phosphate, and required similar concentrations of ATP (0.6 nM) for half-maximal activity. The amount of membrane-bound phosphoenzyme formed from ATP in the presence of Ca\(^{2+}\) was the same regardless of whether one or both sites were saturated, suggesting that occupancy of the second Ca\(^{2+}\) binding site accelerates the enzyme turnover. Inhibition of Ca\(^{2+}\) transport by Na\(^+\) was reversed by the addition of ouabain or an ATP-regenerating system, indicating that this inhibitory effect of Na\(^+\) on Ca\(^{2+}\) uptake may be due to the accumulation of ADP in the medium as a result of Na\(^+\) pump activity. Low concentrations of carbonyl cyanide p-trifluoromethoxyphenylhydrazone and valinomycin (2.5 and 1 \(\mu\)M, respectively) were without effect on Ca\(^{2+}\) uptake in the presence of phosphate, whereas higher concentrations of the ionophores (200 and 100 \(\mu\)M, respectively) reduced uptake by 60% or more. The calmodulin antagonist 48/80 also reduced Ca\(^{2+}\) uptake with half-maximal effectiveness at 100 \(\mu\)g/ml. None of these drugs affected either ATPase activity or the EGTA-induced Ca\(^{2+}\) efflux from preloaded vesicles. The Ca\(^{2+}\) dependence of ATP hydrolysis by the membrane-bound enzyme preparation was similar to that observed for Ca\(^{2+}\) uptake by the vesicles. However, with solubilized enzyme, concentrations of Ca\(^{2+}\) similar to that found in the plasma reduced Ca\(^{2+}\)-stimulated ATP hydrolysis to one-half of its maximal rate. This indicates that peritubular Ca\(^{2+}\) may play a role in the regulation of Ca\(^{2+}\) transport across the tubular epithelium. ATP could not be replaced by ITP as a substrate for Ca\(^{2+}\) uptake, and the (Ca\(^{2+}\) + Mg\(^{2+}\))ITPase activity of soluble enzyme was 25-fold lower than in the presence of ATP. This is an indication that the active Ca\(^{2+}\) pumping mechanism in proximal tubules is critically dependent on the nucleoside moiety of the substrate.

The regulation of Ca\(^{2+}\) concentrations in different body fluid compartments is largely dependent on the control of Ca\(^{2+}\) fluxes across kidney tubular epithelia (1). In mammals, micropuncture studies indicate that Ca\(^{2+}\) is reabsorbed from all parts of the nephron, with the bulk of the reabsorption occurring in the convoluted portion of the proximal tubule (2). Reabsorption of two-thirds of the filtered Ca\(^{2+}\) has been attributed to an overall active transport (2–4) mediated in part by a Ca\(^{2+}\)-dependent ATPase (5–7) located in the antiluminal membrane of tubular cells (6, 8).

In the literature there are controversial data concerning both the Ca\(^{2+}\) sensitivity of the Ca\(^{2+}\)-transport ATPase and whether Mg\(^{2+}\) has a role in ATP hydrolysis that corresponds to its role in Ca\(^{2+}\) transport. The enzyme obtained from kidney cortex was initially described as an ATPase stimulated by Ca\(^{2+}\) or Mg\(^{2+}\) in the millimolar concentration range (6, 6, 9). It was difficult to reconcile these data with the concept of a pumping mechanism involved in transepithelial Ca\(^{2+}\) transport, since the cytosolic Ca\(^{2+}\) concentration (free calcium) is below 1 \(\mu\)M (10). More recently, it has been shown that both the Ca\(^{2+}\) pump and the ATPase activity of kidney tubular membranes are stimulated by micromolar Ca\(^{2+}\) concentrations in the presence of Mg\(^{2+}\) (4, 8, 11, 12).

The present report compares the Ca\(^{2+}\), Mg\(^{2+}\), ATP, and ITP sensitivities of Ca\(^{2+}\) accumulation and the Ca\(^{2+}\)-ATPase activity, describes the effects of phosphate on Ca\(^{2+}\) transport at different pH values, and explores the mechanism by which Na\(^+\) inhibits Ca\(^{2+}\) transport in vitro across the membranes of proximal tubules. We show that there are both a high-affinity and a low-affinity system for Ca\(^{2+}\) transport and that Ca\(^{2+}\) transport requires ATP and Mg\(^{2+}\), is stimulated by phosphate, and is inhibited by ADP and by high concentrations of FCCP, valinomycin, and compound 48/80. With respect to the ATP-

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†The abbreviations used are: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N"-tetrasacetic acid.
ase activity of the membrane-bound enzyme, we find that ATP hydrolysis is also activated by Ca2+ with high- and low-affinity kinetic components, but does not require Mg2+. Formation of phosphoenzyme is maximal when the high-affinity Ca2+ site is saturated. The pattern of activation by divalent cations is different in the Triton X-100-solubilized enzyme, which is activated only by low concentrations of Ca2+ and only in the presence of Mg2+; millimolar concentrations of Ca2+ are inhibitory.

**EXPERIMENTAL PROCEDURES**

**Isolation of Plasma Membrane Fraction—** A modification of the method described by Fitzpatrick et al. (13) was used to prepare a vesicular plasma membrane fraction from the outermost region of rabbit kidney cortex, which is rich in proximal tubules (14, 15). The cortex cortices were dissected from transverse slices using iridectomy scissors and homogenized by hand in a cold-buffered isotonic sucrose medium (pH 7.4) employing a Teflon glass homogenizer (standard pestle, 10 strokes). The homogenate was centrifuged at 1,600 × g for 15 min and the resulting sediment resuspended in 2 mM sucrose. After centrifugation for 20 min at 13,500 × g, the supernatant was immediately diluted to isotonicity with cold deionized water, mixed with one stroke of the homogenizer, and then centrifuged for 30 min at 35,000 × g. The upper layer of the sediment was separated from the overlying supernatant using 1 ml of 0.25 M sucrose, which was used within 2 h. Chelators of divalent cations such as EGTA or EDTA were not used. In some preparations, residual mitochondrial contamination detected in the resuspended upper layer by succinate dehydrogenase assays (16) was removed by further centrifugation at 9,000 × g for 15 min, and the microsomal fraction was recovered by a final centrifugation of the supernatant at 55,000 × g for 40 min. In later experiments, mitochondrial contamination was reduced by a preliminary 20-min centrifugation at 9,000 × g for 10 min, and the microsomal fraction was recovered by a final centrifugation of the supernatant at 55,000 × g for 40 min. In later experiments, mitochondrial contamination was reduced by a preliminary 20-min centrifugation at 9,000 × g for 15 min, and the microsomal fraction was recovered by a final centrifugation of the supernatant at 55,000 × g for 40 min.

**Solubilization of Renal Ca2+-ATPase with Triton X-100—** Solubilization of the enzyme was carried out for 20 min in an ice-cold solution containing 17.2 mg of Triton X-100, 17.2 mg of microsomal protein containing 17.2 mg of Triton X-100, 17.2 mg of microsomal protein and 1 ml of 0.25 M sucrose, which was used within 2 h. Chelators of divalent cations such as EGTA or EDTA were not used. In some preparations, residual mitochondrial contamination detected in the resuspended upper layer by succinate dehydrogenase assays (16) was removed by further centrifugation at 9,000 × g for 15 min, and the microsomal fraction was recovered by a final centrifugation of the supernatant at 55,000 × g for 40 min. In later experiments, mitochondrial contamination was reduced by a preliminary 20-min centrifugation at 9,000 × g for 15 min, and the microsomal fraction was recovered by a final centrifugation of the supernatant at 55,000 × g for 40 min. The supernatant was diluted to a protein concentration of 1 mg/ml in 0.25 M sucrose and used immediately for determinations of the ATPase activity. The pellet was washed once, and it retained ATPase activity with a cation dependence that resembled that of the native vesicular preparation (see "Results and Discussion").

**Reagents and Solutions—** Nucleotides, ouabain, P-enolpyruvate, pyruvate kinase, FCCP, valinomycin, and compound 48/80 were purchased from Sigma, molecular weight standards were from Bio-Rad, and radioisotopes were from the Brazilian Institute of Atomic Energy. All reagents were of analytical grade. Deionized distilled water was used in the preparation of all solutions. The Ca2+ contamination of reagents and solutions was measured by atomic absorption spectroscopy. In Ca/EGTA-buffered solutions, free Ca2+ was calculated using a simple computer program that took into account the different species involved in the equilibria between EGTA, Ca2+, ATP, Mg2+, H+, P-enolpyruvate, and P, and the influence of ionic strength on the association constant for Ca/EGTA (19).

RESULTS AND DISCUSSION

**Effects of Phosphate and ATP on Ca2+ Uptake—** In the absence of precipitating agents, Ca2+ accumulation by plasma
membrane vesicles reached 5 nmol/mg of protein in 1 h when the medium was supplied with ATP (Fig. 1). The ATP-dependent Ca\(^{2+}\) uptake increased more than 20-fold upon addition of 80 mM Pi (Fig. 1). These results are in agreement with those recently obtained with a more purified preparation of basolateral membrane vesicles (24). The bulk of Ca\(^{2+}\) accumulation observed in the present study can be attributed to basolateral membranes present in our preparation, since the contribution of mitochondrial and light microsomal fractions to the observed ATP-dependent Ca\(^{2+}\) accumulation was kept to a minimum (see "Experimental Procedures") by the method of preparing the vesicles and by the use of azide in the uptake medium. Also, Ca\(^{2+}\) uptake under optimal conditions was completely abolished by vanadate (not shown), in contrast with that of endoplasmic reticulum (12).

Several observations suggest that Ca\(^{2+}\) accumulation under the conditions of Fig. 1 is an active transport process. There was minimal accumulation of Ca\(^{2+}\) in the absence of ATP and precipitating anions. In other Ca\(^{2+}\)-transporting membrane preparations, enhancement of Ca\(^{2+}\) uptake by Pi or oxalate has been attributed to decreased Ca\(^{2+}\) outflux as the Ca salts precipitate in the intravesicular space (27, 28). Moreover, in our experiments the Ca\(^{2+}\) accumulated by the vesicles in the presence of Pi was released to the medium after addition of 0.05 mM ADP or 2 mM EGTA, probably due to a decrease of Ca\(^{2+}\) concentration in the medium below the high-affinity \(K_m\) of the Ca\(^{2+}\) pump (see below, Fig. 5A). These experiments show that the accumulated Ca\(^{2+}\) was trapped in the vesicular lumen, generating a Ca\(^{2+}\) concentration gradient across the membrane.

**Opposing Effects of Na\(^{+}\), Ouabain, and an ATP-regenerating System on Ca\(^{2+}\) Accumulation**—When the experiment of Fig. 1 was repeated in a medium containing Na\(^{+}\) phosphate but without ouabain, a significant decrease in Ca\(^{2+}\) uptake was observed (Fig. 2A). This inhibition disappeared when the Na\(^{+}\) in the medium was replaced by K\(^{+}\). The Na\(^{+}\) inhibition observed in the present study confirms previous observations in a similar membrane preparation (7) and in purified basolateral membranes (4, 12). A possible explanation for these two findings is that both conditions, lack of Na\(^{+}\) and presence of ouabain, diminish the (Na\(^{+}\) + K\(^{+}\))ATPase activity that is also present in these membranes, preventing the diminution of the ATP concentration in the medium and limiting the accumulation of ADP. The ouabain-sensitive (Na\(^{+}\) + K\(^{+}\))ATPase activity in our preparation was 15 nmol Pi\(^{-}\)/mg protein, and 20 mU of membrane protein/ml either in the absence (A, B) or in the presence of 5 mM ATP (A, C, D), without addition of phosphate (A, C), or in the presence of 80 mM Na\(^{+}\) phosphate and 12 mM KCl (C, D). The Na\(^{+}\) and K\(^{+}\) concentrations of the different media were maintained equal by addition of appropriate amounts of NaCl or KCl. At the arrow, either 10 µg/ml of ionophore A23187 (○), 2 mM EGTA (○), or 1% (v/v) dimethyl sulfoxide (X) was added. Accumulated Ca was measured by millipore filtration, as indicated under "Experimental Procedures." Values obtained in the presence of ATP were the means of four experiments, with standard errors of 10% or less.

**FIG. 1. Time course of Ca\(^{2+}\) uptake in the presence or absence of ATP and Pi.** Assay media contained 30 mM Tris- HCl buffer (pH 7.0), 10 mM NaNO\(_3\), 0.2 mM ouabain, 0.05 mM \(^45\)CaCl\(_2\), 5 mM MgCl\(_2\), and 0.2 mg of membrane protein/ml either in the absence (A, B) or in the presence of 5 mM ATP (A, C, D), without addition of phosphate (A, C), or in the presence of 80 mM Na\(^{+}\) phosphate and 12 mM KCl (C, D). The Na\(^{+}\) and K\(^{+}\) concentrations of the different media were maintained equal by addition of appropriate amounts of NaCl or KCl. At the arrow, either 10 µg/ml of ionophore A23187 (○), 2 mM EGTA (○), or 1% (v/v) dimethyl sulfoxide (X) was added. Accumulated Ca was measured by millipore filtration, as indicated under "Experimental Procedures." Values obtained in the presence of ATP were the means of four experiments, with standard errors of 10% or less.

**FIG. 2. Effects of different phosphate salts, ouabain, and ATP-regenerating system on Ca\(^{2+}\) uptake.** A, assays contained 30 mM Tris-HCl buffer (pH 7.0), 10 mM NaNO\(_3\), 5 mM MgCl\(_2\), 5 mM ATP, 0.05 mM \(^45\)CaCl\(_2\), and 0.2 mg of membrane protein/ml in the presence of 80 mM Na\(^{+}\) phosphate and 12 mM KCl (without ouabain) (A), 80 mM K\(^{+}\) phosphate (○), 80 mM Na\(^{+}\) phosphate with 12 mM KCl and 0.20 mM ouabain (□), or 80 mM Na\(^{+}\) phosphate and 12 mM KCl with 3 mM P-enolpyruvate, and 0.05 mg/ml pyruvate kinase (●). B, assays containing Tris-HCl, NaN\(_3\), ouabain, MgCl\(_2\), ATP, K\(^{+}\) phosphate, and membrane protein as in A, were preincubated at 37°C for 40 min in the absence (●) or in the presence of 3 mM P-enolpyruvate and 0.05 mg/ml pyruvate kinase (○). Ca\(^{2+}\) accumulation was started at zero time by the addition of 0.05 mM \(^45\)CaCl\(_2\). Values shown are means of four experiments, with standard errors of 10% or less. In A, the difference in the amounts of Ca\(^{2+}\) taken up by the vesicles after 60 min in the media with and without (△) ouabain (134 nmol × mg\(^{-1}\) × 66 nmol × mg\(^{-1}\) × min\(^{-1}\)), respectively) was highly significant (t = 49, n = 4, P < 0.001).

This interpretation is supported by the observation that addition of P-enolpyruvate and pyruvate kinase, which converts to ATP all the ADP formed during hydrolysis, was as effective as ouabain in reversing the inhibition by Na\(^{+}\) (Fig. 2A). This effect was more pronounced when the vesicles were preincubated with ATP, Pi, and MgCl\(_2\) before the addition of \(^45\)CaCl\(_2\) to start Ca\(^{2+}\) uptake (Fig. 2B). The initial velocity and the amount of Ca stored after 40 min were much higher in preincubated vesicles, both in the presence and in the absence of an ATP-regenerating system, than in standard incubation conditions (compare Fig. 2, A and B). We do not have any explanation for this finding at the present.

An inhibitory effect of ADP is seen in Fig. 3. In the absence of the ATP-regenerating system, the addition of exogenous ADP inhibited Ca\(^{2+}\) uptake in a medium without Na\(^{+}\). The effect of ADP on the initial rate of Ca\(^{2+}\) transport was more pronounced at a subsaturating ATP concentration. Thus 0.4 mM ADP decreased the initial rate of uptake by 40% when ATP was 5 mM and 70% when ATP was 0.6 mM (Fig. 3). This suggests that competition between ADP and ATP for a nucleotide binding site on the Ca\(^{2+}\)-ATPase may be responsible for the inhibition of Ca\(^{2+}\) uptake in an initial step of the transport cycle.

The experiments of Figs. 2 and 3, taken as a whole, indicate that the ADP generated in the Na\(^{+}\) transport processes (14, 15) is involved in the inhibition of Ca\(^{2+}\) uptake by Na\(^{+}\).

**Dependence of Ca\(^{2+}\) Uptake on Ca\(^{2+}\) Concentration in Phosphate-containing Media—Initial rates of Ca\(^{2+}\) accumulation by native membrane vesicles in the presence of Pi, increased in a complex way with increasing concentrations of Ca\(^{2+}\) (Fig. 4).**
between 1 and 2 mM EGTA, and enough CaCl₂ to give free Ca²⁺ concentrations varying from 60 to 4250 pM were incubated for different time intervals and initial rates were calculated as indicated under "Experimental Procedures." Initial rates were calculated from the regression line are 251 μM and 18.8 nmol Ca²⁺ (data from principal panel of Fig. 4). Inset, Ca²⁺ activation profile of Ca²⁺ uptake in the concentration range from 1 to 6.1 μM (dashed area in principal figure). Data shown are accurate to within 30% of standard error of the mean.

In order to avoid precipitation of Ca phosphate in the assay medium, Ca²⁺ concentrations higher than 400 μM were not used. Depending on the range of Ca²⁺ concentrations examined, linear Lineweaver-Burk plots with markedly different slopes and intercepts were obtained (Fig. 5). The high-affinity system (Fig. 5A) transported Ca²⁺ at a low rate (Vₘₐₓ ≈ 0.15 nmol × mg⁻¹ × min⁻¹). The Kₘ for Ca²⁺ in this system (1.8 μM) is in the range of cytosolic Ca²⁺ activity (10) and is similar to the Kₘ for Ca²⁺ of the Ca²⁺ pump in purified basolateral membrane vesicles from rat kidney (4, 12). This may indicate that an ATP-dependent transport mechanism contributes to the active reabsorption of Ca²⁺ by proximal tubular epithelium, in addition to a Na-Ca exchange mechanism (29, 30).

In addition to the high-affinity system, the present study revealed a second system with a low affinity for Ca²⁺ (Kₘ = 250 μM). This Kₘ may correspond to the one reported by Moore et al. (7) using the same preparation as in the present study. Since they did not use Ca/EGTA, the true Ca²⁺ concentrations in their assay media, and the calculated Kₘ, were probably higher than they reported. Whether the two affinities we observe are due to two sites in the enzyme or to heterogeneity of the enzyme preparation cannot be decided by these experiments. The fact that both components are inhibited by vanadate (data not shown) favors the hypothesis that they are derived from the same membrane (cf. Ref. 12). It may be speculated that the low-affinity (high-capacity) system for Ca²⁺ extrusion would be operative under conditions in which Na-Ca exchange is compromised (e.g. inhibition of Na⁺ pump) and cytosolic Ca²⁺ activity is increased.

**Phosphorylation by ATP in the Presence of Low and High Ca²⁺ Concentrations and Electrophoresis of Membrane Proteins.** The following experiments were performed to investigate whether the two kinetic systems shown in Fig. 5, A and B, were associated with different steady levels of phosphorylzyme. The results of a set of experiments to measure the levels of phosphorylated intermediate formed in the presence of ATP at different Ca²⁺ concentrations are given in Table I. Formation of a Ca²⁺-dependent phosphoenzyme had been detected in a study of the molecular properties of Ca²⁺-ATPase and alkaline phosphatase in renal plasma membranes (24). However, the authors did not measure directly the amount of §P covalently bound to the enzyme. The values in Table I are in the range of those obtained with red cells (31) or duodenal epithelium (32). They decreased in the presence of hydroxylamine, indicating that the phosphorylzyme formed is an acylphosphate-type compound analogous to that formed during the catalytic cycle of other Ca²⁺-transporting enzymes. The low values of E ~ P were readily expected, since the Ca²⁺-pumping units in these membranes represents only a minor fraction of the proteins in the preparation. The electrophoretic profile indicates that a small portion of the membrane proteins migrate with apparent molecular masses between 100 and 130 kDa (Fig. 6). These are the values reported for the Ca²⁺-ATPases of different plasma membrane preparations (32, 33) including renal tubules (24).

**Table I.** Formation of a Ca²⁺-dependent phosphoenzyme (Table I) are in the range of those obtained with red cells (31) or duodenal epithelium (32). They decreased in the presence of hydroxylamine, indicating that the phosphorylzyme formed is an acylphosphate-type compound analogous to that formed during the catalytic cycle of other Ca²⁺-transporting enzymes. The low values of E ~ P were readily expected, since the Ca²⁺-pumping units in these membranes represents only a minor fraction of the proteins in the preparation. The electrophoretic profile indicates that a small portion of the membrane proteins migrate with apparent molecular masses between 100 and 130 kDa (Fig. 6). These are the values reported for the Ca²⁺-ATPases of different plasma membrane preparations (32, 33) including renal tubules (24).

**Table I. Formation of a Ca²⁺-dependent phosphoenzyme**

<table>
<thead>
<tr>
<th>Ca²⁺</th>
<th>Phosphoenzyme</th>
<th>E ~ P remaining after NH₄OH treatment</th>
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<tr>
<td>μM</td>
<td>pmol/mg protein</td>
<td>pmol/mg protein</td>
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<tr>
<td>0</td>
<td>2.12 ± 0.28</td>
<td>0.10</td>
</tr>
<tr>
<td>10</td>
<td>5.35 ± 0.43</td>
<td>0.20</td>
</tr>
<tr>
<td>500</td>
<td>5.10 ± 0.08</td>
<td>0.22</td>
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VESICLES. The molecular weights of the principal bands of the dodecyl sulfate, were performed as indicated under "Experimental Procedures." The molecular weights of the principal bands of the (Na+ + K+)ATPase (80 µg), isolated as described under "Experimental Procedures." The molecular weights of the principal bands of the (Na+ + K+)ATPase lane (α- and β-subunits) were taken from Ref. 26. Electrophoresis in acrylamide gels in the presence of sodium dodecyl sulfate, were performed as indicated under "Experimental Procedures."

FIG. 6. Polyacrylamide gel electrophoresis of untreated vesicles. Center lane, 250 µg of membrane protein from untreated vesicles. Right lane, standards (2 µg each of myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin. Left lane, (Na+ + K+)ATPase (80 µg), isolated as described under "Experimental Procedures." The molecular weights of the principal bands of the (Na+ + K+)ATPase lane (α- and β-subunits) were taken from Ref. 26. Electrophoresis in acrylamide gels in the presence of sodium dodecyl sulfate, were performed as indicated under "Experimental Procedures."

![Graph of ΔM Ca x mg⁻¹ vs [Pi] (mM) for different pH values](image)

**Fig. 7.** Dependence of Ca²⁺ uptake on Pi concentration at different pH values. A, assays contained NaNO₃, ouabain, MgCl₂, ATP, P-enolpyruvate, pyruvate kinase, CaCl₂, and membrane protein as described in the legend to Fig. 2, 30 mM Tris-HCl buffer and varying concentrations of K⁺ phosphate ranging from 1 to 80 mM at pH 7.0 (○) or pH 8.0 (●). After 60 min aliquots were removed for determination of accumulated Ca₂⁺. B, the assay media and symbols were as in A, except that CaCl₂ was 0.25 mM, P-enolpyruvate was 6 mM, phosphate varied between 2.5 and 120 mM, and incubation time was 120 min. All data points correspond to steady-state levels of Ca²⁺ uptake and are means of four experiments with standard errors of 10% or less.

Identical amounts of phosphoenzyme were formed regardless of whether the phosphorylation was carried out with 10 or 500 µM Ca²⁺. Since the E ~ P level did not increase with increasing saturation of the low-affinity Ca²⁺-binding site shown in Fig. 5B, this could indicate that occupancy of the low-affinity site increases the rate of turnover of the enzyme, without affecting the steady level of phosphorylated intermediates during the catalytic cycle.

**Dependence of Ca²⁺ Transport on P Concentration at Different pH Values**—The effect of P, concentration of Ca²⁺ uptake, not previously determined, was studied at two different pH values (Fig. 7). Pi increased steady-state Ca²⁺ uptake in a dose-dependent manner. The maximal effect was attained in the presence of 80 mM Pi, both at pH 7.0 and 8.0 when the high-affinity Ca²⁺-binding site shown in Fig. 5A was saturated (Fig. 7A). Essentially the same Pi concentration profiles were obtained with half-saturation of the low-affinity Ca²⁺-binding site (Fig. 7B). The steady-state accumulation profile shifted from a sigmoidal dependence on Pi concentration at pH 8.0 to a hyperbolic dependence when the pH was lowered to 7.0.

The higher apparent affinity for Pi, at the lower pH value indicates that the rate-limiting step for Ca²⁺ accumulation within the vesicles may be the diffusion of Pi anions. It is possible that at alkaline pH the diffusion of the predominant HPO₄²⁻ form is more restricted than that of H₂PO₄⁻, leading to less phosphate accumulation inside the vesicles and, consequently, restricted Ca²⁺ accumulation. On the other hand, asymmetrical distribution of different forms of Pi between membrane and intravesicular space may change Ca²⁺ uptake by altering downhill diffusion potentials (34) or by partial compensation of charge transfer via a Ca²⁺/H⁺ antiporter mechanism (35). Differences in the flux of HPO₄²⁻ and H₂PO₄⁻ could generate a proton gradient and/or an electrical diffusion potential. In sarcoplasmic reticulum vesicles it has been found that H⁺ release and Ca²⁺ accumulation occur simultaneously (36) and that an artificial acidification of the vesicular lumen stimulates Ca²⁺ uptake (37). Asymmetrical distribution of different forms of Pi, rather than alterations in total intravesicular Pi concentration, are probably responsible for the observed modulation of Ca²⁺ uptake by Pi at different pH values (Fig. 7).

**Experiments with FCCP, Valinomycin, and Compound 48/80**—Calcium uptake in the presence of 80 mM K⁺ phosphate was inhibited by increasing concentrations of FCCP, which increases membrane permeability to protons, and of valinomycin, which collapses the membrane potential (Fig. 8, A and B, solid symbols). The maximal effects of these inhibitors were attained, however, at concentrations too high to be explained solely on the basis of proton permeability or membrane potential (38). The concentration dependence for both compounds varied with the medium composition. The effect of FCCP was more pronounced when the Pi concentration was lowered to 20 mM (Fig. 8A). On the other hand, the valinomycin inhibition disappeared when Pi was reduced to 20 mM, or when Na⁺ replaced K⁺ (Fig. 8B, open symbols). Thus the effect of valinomycin was K⁺-dependent.

The effects of FCCP and valinomycin might indicate that Ca²⁺ transport is not a primary active process, but is secondary to a proton movement or charge gradient. Fig. 8C shows that Ca²⁺ uptake was inhibited by the calmodulin antagonist 48–80 as previously reported for sarcoplasmic reticulum vesicles (39). Since Ca²⁺ chelators were not used during isolation of the vesicles, our preparation may contain calmodulin. The inhibition by compound 48/80 is consistent with existence of a primary ATP-dependent Ca²⁺ transport process in this preparation.

The possibility that the three inhibitors shown in Fig. 8 act by disrupting the membrane in a nonspecific way, is ruled out by the observations of Fig. 9. The addition of 200 µM FCCP or 500 µg/ml 48/80 did not affect the EGTA-induced Ca²⁺ efflux from vesicles preloaded with Ca in the presence of phosphate. In contrast, the Ca²⁺ ionophore A23187 promoted a rapid and complete release of Ca²⁺ previously taken up by the vesicles. The ATPase activities measured in the presence of 50 µM Ca²⁺, 5 mM MgCl₂, and 5 mM ATP were identical to the absence of the drugs and in the presence of 200 µM FCCP, 100 µM valinomycin, or 500 µg/ml 48/80 (with an average value of 2.2 µmol P · mg⁻¹ · min⁻¹). These results, taken as a whole, seem to indicate that the three compounds assayed in Fig. 8 promote, probably through different mechanisms, inhibition of Ca²⁺ accumulation without changing the Ca²⁺ permeability.

**Dependence of ATPase Activity on Ca²⁺ Concentration in the Micromolar Range**—In the absence of added Mg²⁺, a biphasic pattern of vesicular ATPase activation by Ca²⁺ was observed. Activation by EGTA-buffered Ca²⁺ concentrations...
between 0.02 and 50.6 μM is shown in Fig. 10A. In this range, a plateau was observed in the vicinity of 10 μM Ca++. A double reciprocal plot of the Ca2+-dependent ATPase activity showed a maximum velocity of 0.26 μmol × mg⁻¹ × 24 min⁻¹ and half-saturation at 0.89 μM Ca2+ (Fig. 10B), in reasonable agreement with the Kₐ for the high-affinity transport system shown in Fig. 5A. It is also very similar to that reported for Ca2+-transport using more purified vesicles of basolateral membranes from rat kidney (4, 12).

Dependence of ATPase Activity on Ca2+ Concentrations in the Millimolar Range: Comparison with MgCl2—The effects on ATPase activity of Ca2+ concentrations above 20 μM are illustrated in Fig. 11A and compared with the activation profile obtained in the presence of MgCl2 and EGTA (Fig. 11B). In this concentration range, when only one of the divergent cations was present, the Sₐₕ and maximal velocity for ATP hydrolysis by the membrane-bound enzyme were similar for CaCl2 and MgCl2. The cation concentration dependence was in both cases rather complex and could not be fitted with a straight line on a double reciprocal plot. In the presence of a high MgCl2 concentration (6 mM), the addition of CaCl2 did not produce any additional stimulation of the ATP hydrolysis (upper curve in Fig. 11A). Furthermore, at lower concentrations of MgCl2 there was also no additive effect of CaCl2 in the presence of 0.07 mM CaCl2 (enough to saturate the high-affinity site seen in Fig. 10) and 0.25 mM MgCl2, the amount of Pi released was identical to that obtained with 0.25 mM MgCl2 or 0.07 mM CaCl2 alone (not shown). These data suggest that Ca2+ and Mg2+ act at the same low-affinity binding site.

Since ITP differs in several respects from ATP as an energy-donor substrate for the Ca2+-ATPase of sarcoplasmic reticulum membranes (40, 41), we measured ITPase activity of the native vesicles from kidney tubules. Its cation dependence was studied in the range from 0.05 to 10 mM of either CaCl2 or MgCl2 (Fig. 11, C and D). Both Vₐₕ and Sₐₕ for CaCl2 and MgCl2 were essentially the same as those observed in the presence of ATP.

Dependence of Ca2+ Uptake on MgCl2 and ATP Concentrations—It has been known for a number of years that Mg2+ is required for Ca2+ uptake by kidney membranes (7, 42). As in other ATP-dependent reactions, Mg2+ is an obligatory cofactor in the catalytic cycle of Ca2+-transporting systems from different sources (28, 43, 44). However, the dependence of Ca2+ accumulation on the concentrations of MgCl₂ and ATP has not previously been reported. In the absence of added MgCl₂, a low level of Ca2+ accumulation was observed. In a medium containing enough Ca2+ to saturate the high-affinity Ca2+ transport site, steady-state Ca2+ uptake reached its maximal value in the presence of 2 mM MgCl2 (Fig. 12A). At a saturating concentration of ATP, half-maximal stimulation of Ca2+ uptake was obtained with 0.4 mM MgCl2, a value that is similar to that obtained for activation of ATP hydrolysis (Fig. 11B). At saturating MgCl2 concentrations the corresponding Sₐₕ value for ATP dependence of Ca2+ uptake was 0.6 mM (Fig. 12B), which corresponds to the high Kₐ for ATP of renal Ca2+-ATPase activity as previously reported (45).

The foregoing experiments showed that the kinetic behavior of ATPase activity and Ca2+ transport towards MgCl2 were essentially the same. On the other hand, Fig. 10A shows that ATP hydrolysis reached 0.2 μmol × mg⁻¹ × 24 min⁻¹ in the presence of 50 μM CaCl2 with no Mg2+ added. If it were coupled to Ca2+ transport this value would be large enough to support the maximal Ca2+ uptake rates observed, e.g. in Figs. 1 and 24. It may be that MgCl2 does not stimulate Ca2+-
transport directly but rather promotes the coupling between ATP hydrolysis and active Ca\(^{2+}\) transport across basolateral membranes of renal tubules. Such behavior would be similar to that observed with T tubule membranes isolated from skeletal muscle. Transverse tubular vesicles display ATPase activity that is stimulated by either Ca\(^{2+}\) or Mg\(^{2+}\), but rather promotes the coupling between the two transport processes (see "Experimental Procedures"). Data points show the means of at least four experiments, with standard errors of 10% or less. B, double reciprocal plot of ATPase activity against Ca\(^{2+}\) concentration in the range from 0.12 to 6.1 \(\mu\)M. \(K_\text{m}\) and \(V_\text{max}\) calculated from the regression line are 0.89 \(\mu\)M and 0.26 \(\mu\)mol P\(_i\) \(\times\) mg\(^{-1}\) \(\times\) 24 min\(^{-1}\), respectively.

**Fig. 10.** Dependence of ATPase activity on Ca\(^{2+}\) concentrations in the micromolar range. **A**, assays containing 30 mM Tris-HCl buffer (pH 7.0), 10 mM Na\(_2\)ATP, 0.2 mM ouabain, 160 mM sucrose, 20 mM KCl, 3 mM P-enolpyruvate, 0.05 mg/ml pyruvate kinase, 5 mM ATP, 0.2 mM EGTA, enough CaCl\(_2\) to give free Ca\(^{2+}\) concentrations between 0.02 and 50.6 \(\mu\)M and 0.3 mg of membrane protein/ml were incubated for 24 min in the absence of added Mg. Release of P\(_i\) was measured colorimetrically and preliminary experiments showed that the reactions were linear against time (see "Experimental Procedures"). Data points show the means of at least four experiments with standard errors of 10% or less. **B**, double reciprocal plot of ATPase activity against Ca\(^{2+}\) concentration in the range from 0.12 to 6.1 \(\mu\)M. \(K_\text{m}\) and \(V_\text{max}\) calculated from the regression line are 0.89 \(\mu\)M and 0.26 \(\mu\)mol P\(_i\) \(\times\) mg\(^{-1}\) \(\times\) 24 min\(^{-1}\), respectively.

**Fig. 11.** Dependence of ATPase and ITPase activities on CaCl\(_2\) and MgCl\(_2\) concentrations. Assay media containing Tris-HCl buffer (pH 7.0), Na\(_2\)ATP, ouabain, sucrose, KCl, P-enolpyruvate, pyruvate kinase, and membrane protein as described in the legend to Fig. 10, and 5 mM ATP (A and B) or 5 mM ITP (C and D) were incubated for 20 min in the presence of various CaCl\(_2\) or MgCl\(_2\) concentrations. A and C, CaCl\(_2\) varying between 0.05 and 10 mM in the absence of added Mg (0) or between 0.05 and 2 mM in the presence of 5 mM MgCl\(_2\) (●). B and D, MgCl\(_2\) varying between 0.25 and 10 mM in a medium containing 0.2 mM EGTA. Measurement of P\(_i\) release as indicated under "Experimental Procedures." Values shown are means of at least four experiments, with standard errors of 10% or less.

**Fig. 12.** Dependence of steady-state Ca\(^{2+}\) accumulation on MgCl\(_2\) and ATP concentrations. **A**, assay media containing Tris-HCl buffer (pH 7.0), Na\(_2\)ATP, ouabain, K\(^+\) phosphate, ATP, P-enolpyruvate, pyruvate kinase, \({ }^{42} \text{CaCl}_2\), and membrane protein as described in the legend to Fig. 2 were incubated for 60 min in the presence of 5 mM ATP and various MgCl\(_2\) concentrations from zero (no added MgCl\(_2\)) to 5 mM. In B, MgCl\(_2\) was kept constant at 5 mM and ATP was varied from 0.1 to 5 mM. In C, MgCl\(_2\) was 5 mM, CaCl\(_2\) was 0.25 mM, P-enolpyruvate was 6 mM and the incubation time was 120 min. Data show means of at least four experiments, with standard errors of 10% or less.

the medium was higher than when only the high-affinity site was saturated, but the \(S_\text{max}\) for ATP was similar in both cases (compare Fig. 12, B and C). This observation, together with the P\(_i\) dependence profile of Ca\(^{2+}\) uptake (Fig. 7) and phosphoenzyme measurements (Table I), reinforces the view that saturation of the second Ca\(^{2+}\) binding site increases the turnover of the enzyme and, thus, the overall rate of Ca\(^{2+}\) transport.

**Dependence of ATPase Activity on Ca\(^{2+}\) Concentration after Treatment with Triton X-100**—The pattern of activation by divalent cations changed completely when the vesicles were solubilized with Triton X-100 (Fig. 13B). In the presence of 10 mM MgCl\(_2\), ATP hydrolysis by the supernatant of the detergent-treated membranes (see "Experimental Procedures") was maximally stimulated by micromolar Ca\(^{2+}\) concentrations, whereas activation by Ca\(^{2+}\) was not observed when MgCl\(_2\) was omitted (Fig. 13B). The double reciprocal plot of Ca\(^{2+}\)-stimulated ATPase activity against Ca\(^{2+}\) concentrations between 0.14 and 10 \(\mu\)M indicated a \(K_\text{m}\) for Ca\(^{2+}\) of 0.56 \(\mu\)M (Fig. 13C), i.e. essentially the same as that shown in Fig. 10B for the untreated membranes in the presence of Ca\(^{2+}\) and no added Mg\(^{2+}\). This value is also very similar to the \(K_\text{m}\) for Ca\(^{2+}\) calculated from the double reciprocal plot of Ca\(^{2+}\)-stimulated ATPase activity against Ca\(^{2+}\) concentrations between 0.14 and 10 \(\mu\)M.
Ca\(^{2+}\) Transport and ATPase Activity in Proximal Tubules

Fig. 13. Dependence of ATPase activity on Ca\(^{2+}\) concentration after treatment with Triton X-100.

A, assay media containing Tris-HCl buffer (pH 7.0), Na\(_2\)SO\(_4\), ouabain, sucrose, KCl, P-enolpyruvate, and pyruvate kinase as described in the legend to Fig. 11, 5 mM ATP, 0.2 mg of membrane protein/ml from the pellet of Triton X-100-treated membranes and various concentrations of CaCl\(_2\) (●, ●) or MgCl\(_2\) plus 0.2 mM EGTA ( ○) and were incubated for 20 min; •, assays in the presence of 10 mM MgCl\(_2\) and the CaCl\(_2\) concentrations indicated in the abcissa. P, release was measured colorimetrically as indicated under “Experimental Procedures.” B, the assays contained Tris-HCl buffer, Na\(_2\)SO\(_4\), ouabain, sucrose, and KCl as in Fig. 11, 5 mM [\(^{32}\)P]ATP, 10 mM MgCl\(_2\), 0.2 mM EGTA, enough CaCl\(_2\) to give free Ca\(^{2+}\) concentrations between 0.01 mM and 10 mM, and 0.5 mg of solubilized protein/ml (supernatant of the pellet used in A). Assays were incubated for 20 min in the absence of added Mg\(^{2+}\) or in the presence of 10 mM MgCl\(_2\) (●). Release of [\(^{32}\)P]Pi during the linear phase of ATP hydrolysis was measured as indicated under “Experimental Procedures.” The values represented by closed circles were obtained after subtraction of the mean value observed in the presence of 10 mM MgCl\(_2\) and 1 mM EGTA (0.31 ìmol P\(_{i}\) × mm\(^{-1}\) × 18 min\(^{-1}\)), C, double reciprocal plot of ATPase activity against Ca\(^{2+}\) concentration in the stimulation phase of ATP hydrolysis shown in panel B. K\(_{m}\) and V\(_{max}\) calculated from the regression line are 0.56 ìmol P\(_{i}\) and 0.85 ìmol of P\(_{i}\) × mm\(^{-1}\) × 18 min\(^{-1}\), respectively. The data in A and B are the means of at least four experiments with standard errors of 10% or less.

Table II

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Nucleotide</th>
<th>Ca(^{2+}) uptake (60 min)</th>
<th>ATPase activity (20 min)</th>
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</thead>
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<tr>
<td>Membrane-bound (untreated)</td>
<td>ATP</td>
<td>134</td>
<td>2250</td>
</tr>
<tr>
<td>Membrane-bound (untreated)</td>
<td>ITP</td>
<td>0</td>
<td>2440</td>
</tr>
<tr>
<td>Soluble</td>
<td>ATP</td>
<td>780</td>
<td>34</td>
</tr>
<tr>
<td>Soluble</td>
<td></td>
<td></td>
<td>34</td>
</tr>
</tbody>
</table>

These values were the same in the absence of added CaCl\(_2\).

for Ca\(^{2+}\) of the high-affinity Ca\(^{2+}\)-transport system revealed in Fig. 5A.

The data of Fig. 13, B and C, showing Ca\(^{2+}\) activation of the hydrolytic activity of the solubilized enzyme in the presence of 10 mM MgCl\(_2\) were plotted after subtracting the activity in the presence of EGTA, which was 0.3 ìmol × mm\(^{-1}\) × 18 min\(^{-1}\) (see legend to Fig. 13). This basal value is 1 order of magnitude lower than that observed under the same condition in the untreated membranes (Fig. 11B), and in fact the majority of the Ca-independent activity observed in Fig. 11B remained in the pellet (Fig. 13A, open circles). In contrast, Mg\(^{2+}\)-dependent Ca\(^{2+}\)-stimulated activity, which was undetectable in the pellet and in untreated vesicles (activity was maximal with Mg\(^{2+}\) alone; see Figs. 11A and 13A), appeared in the supernatant with a V\(_{max}\) of 0.8 ìmol of P\(_{i}\) × mm\(^{-1}\) × 18 min\(^{-1}\) (Fig. 13C). This large increase in Mg\(^{2+}\)-dependent Ca\(^{2+}\)-stimulated ATPase activity in the supernatant may be ascribed to a purification process (8, 11) with a concomitant decrease of the activity stimulated by millimolar concentrations of either CaCl\(_2\) or MgCl\(_2\) (Fig. 11, A and B) in native vesicles.

Conversely, the Ca\(^{2+}\) dependence in the millimolar concentration range observed for intact vesicles (Fig. 11A) completely disappeared when the enzyme was solubilized, regardless of whether Mg\(^{2+}\) was present or absent. Raising the Ca\(^{2+}\) concentration above 100 ìM led to a progressive inhibition of ATPase activity, with an I\(_{50}\) value of about 2.5 mM Ca\(^{2+}\) (Fig. 13B). This value is in the range of plasma and peritubular Ca\(^{2+}\) concentrations. Therefore it is reasonable to postulate that the activity of Ca\(^{2+}\)-ATPase is modulated by variations in plasma Ca\(^{2+}\) concentrations. If this is the case, plasma Ca\(^{2+}\) concentration may influence the overall transtubular transport of Ca\(^{2+}\) by a direct action on the basolateral Ca\(^{2+}\)-ATPase activity. This may be achieved through the binding of Ca\(^{2+}\) to a low-affinity and inhibitory Ca\(^{2+}\) binding site oriented toward the interstitial space.

The observation that Ca\(^{2+}\) inhibits ATPase activity in the solubilized preparation but not in intact vesicles is a puzzling one. It may be that inhibition of the low-affinity ATPase activity also exists in intact vesicles, but cannot be observed because it is masked by the activity linked to the low-affinity Ca\(^{2+}\) site shown in Fig. 11A. In the solubilization process, this latter site seems to remain in the pellet, since both the cation dependence and the maximal specific activity of the ATPase in this fraction (Fig. 13A) resembled that previously shown in Fig. 11, A and B, with the native preparation.

The existence of two functionally different ATPase activities, only one being coupled to Ca\(^{2+}\) transport, has been demonstrated in sarcoplasmic reticulum membranes (47), and the effect of detergent solubilization on their cation and...
substrate dependence have been documented (48). It is not clear at present whether the Mg²⁺- or Ca²⁺-dependent activities exhibited by the renal membrane-bound enzyme (Fig. 11) and the (Ca²⁺ + Mg²⁺)ATPase activity measured in the supernatant (Fig. 13B) correspond to two different enzymes, or whether the two activities arise from two different forms of the same enzyme.

Nucleotide Specificity of Ca²⁺ Uptake and Hydrolytic Activity—Since the untreated kidney proximal tubular membranes were capable of hydrolyzing ITP, (Fig. 11, C and D), we attempted to measure Ca²⁺ uptake in the presence of this nucleotide. In contrast with other Ca²⁺-transporting membranes (49), this preparation did not accumulate Ca²⁺ in the presence of 5 mM ITP even when the assay medium contained 80 mM Pi and a nucleotide-regenerating system (Table II). This suggests that active Ca²⁺ transport across the proximal tubular epithelium strongly depends on the nucleoside moiety of the substrate. The (Ca²⁺ + Mg²⁺)ITPase activity remaining in the supernatant after treatment with Triton was less than 5% of that of the untreated membranes (Table II). This finding supports the view that the enzyme form associated with Ca²⁺ translocation across the basolateral membrane is solubilized by Triton treatment, and does not correspond to that which in native vesicles (Fig. 11) catalyzes the hydrolysis of ATP and ITP in the presence of either Ca²⁺ or Mg²⁺.

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