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PROPERTIES OF A Ca^{2+}- AND Mg^{2+}-ACTIVATED ATP-HYDROLYZING ENZYME IN RAT KIDNEY CORTEX

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SUMMARY

1. The properties of an ATP-hydrolysing enzyme (ATP phosphohydrolase, EC 3.6.1.3) in rat kidney cortex, activated by bivalent cations, have been defined.

2. This ATPase is activated by both Ca^{2+} and Mg^{2+}; but Mg^{2+} is not necessary for Ca^{2+} activation.

3. Neither Na^+ nor K^+ are necessary for Ca^{2+} activation.

4. Inhibition occurs at a bivalent cation concentration above 5 to 10 mM.

5. GTP and ITP are also hydrolysed by the enzyme but not ADP.

6. Mn^{2+} but not Sr^{2+} may also stimulate hydrolysis of ATP.

7. The pH optimum for activation lies between 7.5 and 8.2.

8. Ouabain (1 mM) and ethacrynic acid (1 mM) do not inhibit activation by Ca^{2+}.

9. The stimulation of ATP hydrolysis by both Ca^{2+} and Mg^{2+} suggests that the enzyme may be involved in the transport of bivalent cations in the renal tubule.

INTRODUCTION

The ionic requirements of the well known Mg^{2+}-activated Na^+, K^+-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) have been defined by many workers[1-5]. All tissues investigated seem to contain a Na^+, K^+-dependent ATPase[6] and it has been suggested that the major proportion of sodium transport is effected through it[1]. (Na^+, K^+)-ATPase accounts for a varying percentage of the total ATPase activity in cell membranes[1]. By using ouabain an ouabain-insensitive portion of ATPase activity has been separated which has been called "Mg^{2+}-dependent ATPase"[1]. This ATPase may be responsible for Ca^{2+} transport in the red cell[7] and in intestinal mucosa[8,9]. The ionic requirements of this "Mg^{2+}-dependent ATPase" in intestinal mucosa have not been defined.

As the kidney is involved in the reabsorption and excretion of calcium and magnesium we investigated the Ca^{2+} and Mg^{2+} stimulation of an ATPase in cell membranes isolated from rat kidney cortex. We established that an ATPase is present which is stimulated by both Ca^{2+} and Mg^{2+}. We report also the ionic requirements and conditions for maximal stimulation of this ATPase.

MATERIALS AND METHODS

Male Wistar rats, of 200 g body weight, fed a regular laboratory chow and tap water *ad libitum*, were killed by decapitation and the kidneys rapidly removed and placed in ice-cold saline. Sections of kidney cortex were homogenized in 30 vol. of a solution containing 87 g sucrose, 1.169 g NaCl, 1.860 g Na_2EDTA, 0.2 g $MgCl_2 \cdot 6H_2O$, and 0.68 g imidazole per l. Ten strokes at 1500 rev./min with a Thomas tissue grinder, Size C, and Teflon pestle, were used for homogenization. We then treated the material by the method of POST AND SEN[10] except for omitting the urea stage.

Samples of the homogenate were then assayed for ATPase activity. An appropriate amount (0.1 ml) of the final homogenate, containing 10–20 μg of protein, was incubated in 1.0 ml mixtures containing 70 mM or 20 mM Tris buffer (pH 7.6), and 5 mM Na_2ATP (adjusted to pH 7.6 with NaOH) and varying concentrations and combinations of Ca^{2+} (as $CaCl_2$), Mg^{2+} ($MgCl_2$), and additional Na^+ (NaCl) for a period of 30 min. The reaction was then terminated by the addition of 1.0 ml icecold 10% (w/v) trichloroacetic acid and by plunging the tubes immediately into an ice–water bath where they were kept until estimation of inorganic phosphate concentration by a modification of the GOMORI[11] method in an AutoAnalyzer. The colour development and inevitable hydrolysis of a small proportion of the ATP was kept constant by adding samples to the sampler plate of the AutoAnalyzer exactly 1 min before sampling started. The amount of hydrolysis was checked initially in every experiment by running controls of ATP in 10% trichloroacetic acid. All samples were run in duplicate after an initial check on reproducibility (see RESULTS). The protein concentration of the membrane preparation was determined by the method of LOWRY *et al.*[12], modified by EGGSTEIN AND KREUTZ[13]. The enzyme activity was expressed at μmoles phosphorus produced per mg of protein in 30 min (P_i μmoles/mg per 30 min), after subtraction of a blank containing only Tris buffer, ATP and 0.1 ml enzyme preparation. To eliminate the possibility that ions already present in the enzyme preparation enhance ATPase activity, Ca^{2+}, Mg^{2+}, and Na^+ determinations were performed before and after allowing the preparation to stand for 2 h at room temperature in $5 \cdot 10^{-4}$ M imidazole–histidine, 5 mM Tris–EDTA buffer (pH 7.4). No measurable amounts were found before or after the procedure. All experiments were performed at least 4 times and the results expressed as mean $\pm$ S.E.

The Ca^{2+} selective electrode was delivered by Orion Research Inc., Cambridge, Mass., U.S.A.

RESULTS

Incubation with Ca^{2+}

Samples of the enzyme preparation were incubated in solutions containing Ca^{2+} concentrations ranging from 0.025–10 mM. In a preliminary series of experiments Na^+, up to a concentration of 70 mM, was added to the incubation solutions. The concentration of Tris buffer (pH 7.6) was also varied from 10–70 mM. Added Na^+ and variations in the concentration of Tris had no effect on the Ca^{2+}-sensitive ATPase activity at optimal Ca^{2+} concentration (4.0–5.0 mM). However, at lower Ca^{2+} concentrations (1.0 mM) Ca^{2+}-ATPase activity was consistently lower in 70 mM Tris whether

or not Na^+ had been added. This can be seen in Table I where incubations in 20 mM or 70 mM Tris are compared at 1 mM and 5 mM Ca^{2+}.

The incubation solution for all subsequent experiments was 20 mM Tris (pH 7.6), 70 mM Na^+ and 5 mM Na_2ATP. Ca^{2+}-sensitive ATPase was also measured in preparations kept at 0 or $-20°$ for varying periods of time. Since activity decreased with increasing age of the preparation, even if kept at $-20°$, we compared activation of ATPase activity by Ca^{2+} with that of other bivalent cations in preparations made on the same day and stored for the same period of time.

In Fig. 1 Ca^{2+} activation of ATP hydrolysis is plotted against calcium concentration. As can be seen, Ca^{2+} activation of the enzyme occurs in concentrations as low as 0.025 mM and slight inhibition occurs above 5 mM. The apparent K_m for calcium determined by using a Woolf plot of $[S]/v$ *versus* $[S]$, was found to be 1.5 mM.[14]

Mg^{2+} may also stimulate enzyme activity. Samples of homogenate were incubated with solutions containing 20 mM Tris (pH 7.6) *plus* 70 mM Na^+. The optimal

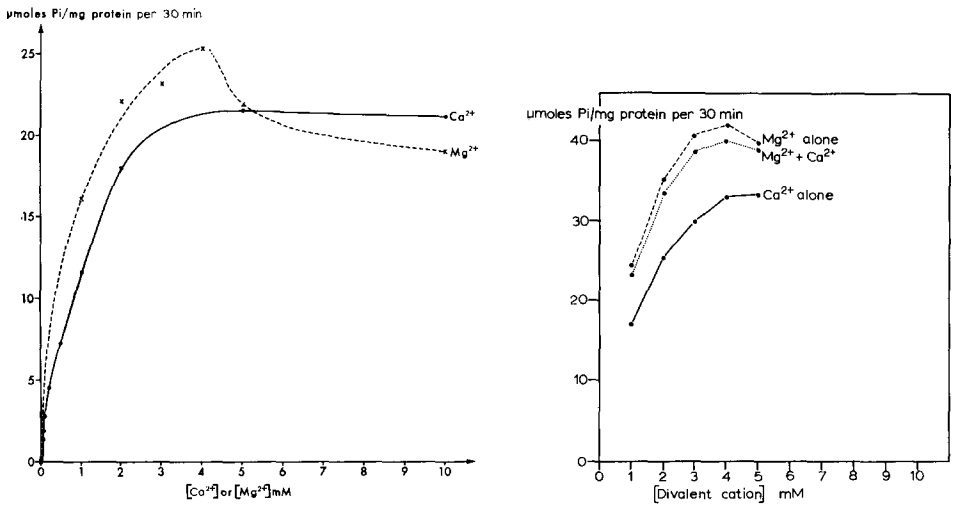


Fig. 1. Stimulation of ATP hydrolysis by Ca^{2+} (●—●) and Mg^{2+} (×- - -×). Incubation solution: 20 mM Tris (pH 7.6) + 70 mM Na^+ + Ca^{2+} or Mg^{2+}, and 5 mM Na_2ATP. Curves are obtained with two different enzyme preparations.

Fig. 2. Stimulation of ATP hydrolysis by Ca^{2+} (●—●), Mg^{2+} (●- - -●) or Ca^{2+} + Mg^{2+} (●· · · · ·●). Conditions otherwise as in Fig. 1. Curves obtained with the same enzyme preparation.

Mg^{2+} concentration for maximal enzyme activity was between 3 and 4 mM; at higher concentrations inhibition occurred (Fig. 1). The maximum activity was higher than with Ca^{2+} alone. The apparent K_m for Mg^{2+} was found to be 1.2 mM.

Incubation with Ca^{2+} plus Mg^{2+}

A comparison with the same enzyme preparation was made between Ca^{2+} alone, Mg^{2+} alone, and Mg^{2+} *plus* Ca^{2+} together in solutions with a total bivalent cation concentration equal to that of Ca^{2+} or Mg^{2+} alone. All solutions contained 20 mM Tris (pH 7.6), 70 mM Na^+ and 5 mM Na_2ATP. Either Ca^{2+} alone or Mg^{2+} alone

stimulated ATPase activity (Fig. 2), usually Mg^{2+} to a greater extent than Ca^{2+}. Activity in the Ca^{2+} *plus* Mg^{2+} solutions, when expressed as activation by total bivalent cation, gave similar curves lying between those of Ca^{2+} or Mg^{2+} alone. This demonstrates that Ca^{2+} or Mg^{2+} may stimulate ATPase activity in the absence of the other. The differences between the curves at their maxima were compared using Student's t test and were significant at the 99% confidence level.

The effect of Mg^{2+} on the Ca^{2+} activation of ATPase was investigated in the following way. Mg^{2+} was added at a concentration of 5 mM to varying concentrations

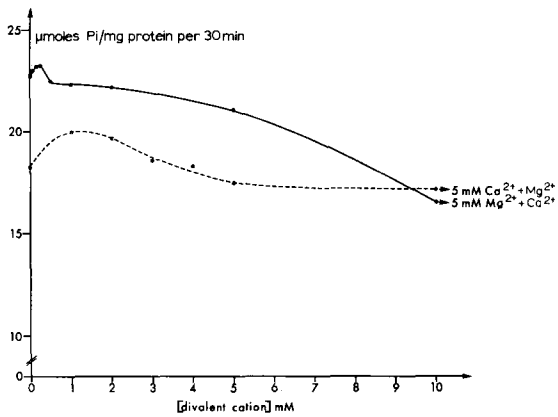


Fig. 3. Effect of addition of Ca^{2+} to a solution containing 5 mM Mg^{2+}, 20 mM Tris (pH 7.6) + 70 mM Na^+ + 5 mM Na_2ATP (●—●) and effect of addition of Mg^{2+} to a solution containing 5 mM Ca^{2+} + 20 mM Tris (pH 7.6) + 70 mM Na^+ + 5 mM Na_2ATP (●----●).

of Ca^{2+} in the usual incubation solution. At this Mg^{2+} concentration, the stimulation of ATPase activity by Ca^{2+} occurred only at a Ca^{2+} concentration between 0.025 and 0.5 mM, as shown in Fig. 3. However, the additional Ca^{2+} activation was seen in all preparations tested.

When Ca^{2+} concentrations were constant at 5 mM and Mg^{2+} was added in concentrations ranging from 1 to 5 mM, slight activation occurred at 1 mM Mg^{2+} and then inhibition was observed at all higher concentrations (Fig. 3).

To test the effect of Na^+ on Ca^{2+} + Mg^{2+} activation, samples were incubated

TABLE I

EFFECT OF TRIS OR Na^+ ON Ca^{2+} ACTIVATION OF ENZYME

P_i (results in μmoles/mg protein per 30 min) at Ca^{2+} concentrations of 1 or 5 mM. Effect of 20 and 70 mM Tris (Mean ± S.E.), and of 0 and 70 mM Na^+.

	1 mM Ca^{2+}	*5 mM Ca^{2+}*
20 mM Tris	14.23 ± 0.40	29.72 ± 0.56
	$P < 0.01$	Not significant
70 mM Tris	12.11 ± 0.34	29.63 ± 0.52
0 mM Na^+	8.56	17.91
70 mM Na^+	8.35	16.58

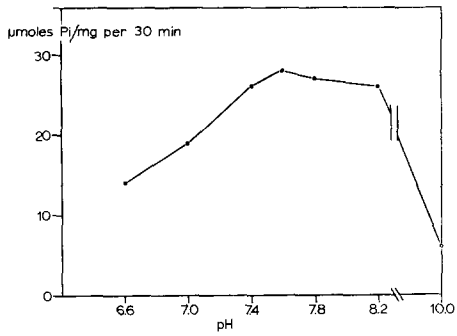


Fig. 4. Effect of pH on enzyme activity. Incubation solution contained 5 mM Ca^{2+}, 20 mM Tris, 70 mM Na^+, and 5 mM Na_2ATP.

with 5 mM Tris ATP, varying concentrations of Ca^{2+} (0–5 mM) and with 0 and 70 mM Na^+. Comparison of the resultant curves showed no significant differences. Table I gives representative results at 1 and 5 mM Ca^{2+} in the presence or absence of Na^+.

Time of incubation

Enzyme preparations were incubated in a solution containing 5 mM Ca^{2+}, 20 mM Tris, 70 mM Na^+ and 5 mM Na_2ATP for periods of 0, 15, 30 and 45 min. The production of phosphate is entirely linear over this time interval.

Temperature

Samples of homogenate were incubated at 25° and 37° in 5 mM Ca^{2+}, 20 mM Tris and 70 mM Na^+. The Q_{10} calculated according to the method of GIESE[15] was 1.8.

pH effect

Similar incubations were performed at pH 6.6, 7.0, 7.4, 7.8, 8.2 and 10.0. The pH optimum is at 7.60 at 37° with a plateau extending from pH 7.4–8.2 (Fig. 4).

Effect of ouabain and ethacrynic acid

Solutions containing 1 or 5 mM Ca and 20 mM Tris *plus* 70 mM Na^+ were incubated in the presence of either ouabain or ethacrynic acid, each 1 mM. The resultant curves were identical and are not shown. No inhibition of activity could be demonstrated with either compound (Table II).

TABLE II

EFFECT OF INHIBITORS, OUABAIN (1 mM) AND ETHACRYNIC ACID (1 mM) ON Ca^{2+}-SENSITIVE ATPASE ACTIVITY

Results expressed as μmoles P_i/mg protein per 30 min. Incubation solutions contained 5 mM Ca^{2+}, 20 mM Tris (pH 7.6), 70 mM Na^+ and 5 mM Na_2ATP (Mean ± S.E.).

	Ouabain	*Ethacrynic acid*
Absence of inhibitor	21.94 ± 0.32	20.34 ± 0.97
Presence of inhibitor	22.00 ± 0.32	18.58 ± 0.97

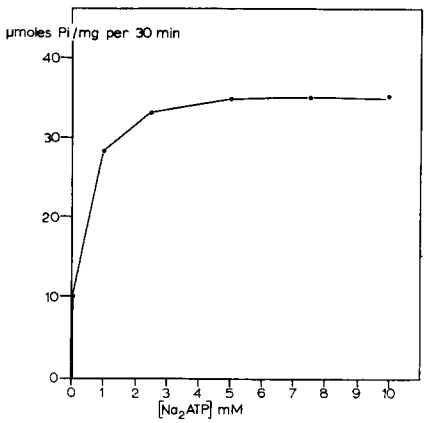


Fig. 5. Plot of ATP concentration *vs.* P_i production.

ATP concentration

Incubations were carried out in 5 mM Ca^{2+} *plus* 20 mM Tris (pH 7.6) *plus* 70 mM Na^+ with differing concentrations of Na_2ATP. The maximum concentration of ATP required for full enzyme activity at 30 min was between 1 and 2 mM (Fig. 5). The K_m for ATP was 0.2 mM.

Other substrates

The specificity of ATP as the substrate for Ca^{2+}-sensitive ATPase was checked by performing similar incubations with the disodium salts of GTP and ITP, each in 5 mM concentrations. Table III shows that both these high energy phosphate compounds are hydrolyzed by the Ca^{2+}-sensitive enzyme but the degree of hydrolysis is less with GTP than with ATP or ITP. Adenosine diphosphate was also used as substrate but no P_i production could be demonstrated using up to 5 mM Ca^{2+}.

Activation by other bivalent cations

Incubations were performed with other bivalent cations, Sr^{2+} and Mn^{2+}, substituting for Ca^{2+} or Mg^{2+}. The results and comparisons with Ca^{2+} are shown in Fig. 6. As can be seen Mn^{2+} has quite a powerful stimulatory effect on ATP hydrolysis with maximal stimulation between 2.0 and 3.0 mM Mn^{2+}. The apparent K_m for Mn^{2+} was 1.9 mM. Sr^{2+}, on the other hand, does not produce ATP hydrolysis.

TABLE III

SUBSTRATE SPECIFICITY: HYDROLYSIS OF OTHER HIGH ENERGY TRIPHOSPHATE COMPOUNDS

Samples contained 5 mM Ca^{2+}, 20 mM Tris (pH 7.6) and 70 mM Na^+. Results expressed as μmole P_i/mg protein per 30 min. Concentration of ITP, GTP or ATP is 5 mM. (Mean ± S.E.; $n = 8$ in each group.)

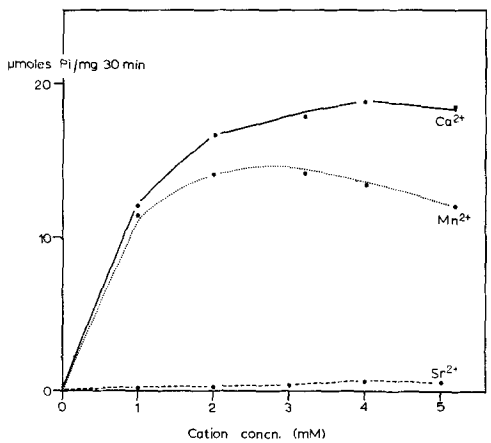
ITP	*ATP*	*GTP*
20.07 ± 0.61	19.65 ± 0.39	14.87 ± 0.49
Not significant		$P < 0.001$

Reproducibility

The coefficient of variation was calculated in seven experiments using different Ca^{2+} concentrations ranging from 0.5 to 5 mM. Eight replicate estimations were performed for each Ca^{2+} concentration. The coefficient of variation ranged from 1.2 to 3.0% with a mean of 2.7%.

Stability of enzyme preparation

To determine the best mode of storage of the renal cortical cell membrane preparation, aliquots of the original solution were frozen immediately and stored at −20° for 7 days or were kept refrigerated throughout the 7-day period. The specimens



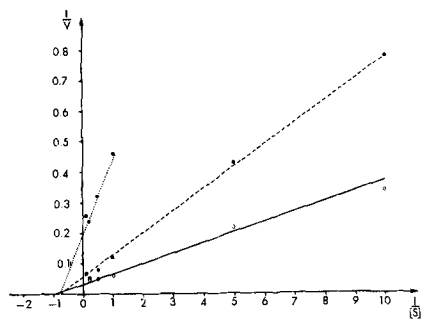


Fig. 6. Comparison of ATPase activation by Ca^{2+} (———), Mn^{2+} (· · · · · ·), and Sr^{2+} (- - - -).

Fig. 7. Lineweaver–Burk plot of enzyme preparations assayed fresh (———) and stored at −20° (· · · · · ·) and +4° (- - - -) for 7 days. Incubation solutions contained 5 mM Ca^{2+}, 20 mM Tris, 70 mM Na and 5 mM Na_2ATP.

were analyzed on the day of preparation and 7 days later. Fig. 7 shows a Lineweaver–Burk plot of the specimens before and after storage. As can be seen the activity of the specimens decreased after storage but the apparent K_m was unchanged.

DISCUSSION

We demonstrated an ATPase sensitive to Ca^{2+} in the plasma membrane of rat kidney cortex. This activity is demonstrable in the presence of Ca^{2+} by itself. The enzyme is also sensitive to Mg^{2+}; our results suggest that the sensitivity to Ca^{2+} or Mg^{2+} is similar in magnitude but that Mg^{2+} always gives higher activation. Several workers have reported that Ca^{2+} can replace Mg^{2+} in activating the ouabain-insensitive component of ATPases[16–18]. Only in the red cell have the ionic requirements been satisfactorily defined by WINS AND SCHOFFENIELS[19], and even here it is not certain if one or more Ca^{2+}-sensitive ATPases are present. Recently, ROSENTHAL *et al.*[20] isolated a Ca^{2+}-sensitive ATPase from erythrocytes which was Ca^{2+}-activated and Mg^{2+}-inhibited. In the kidney, WHEELER AND WHITTAM[21] demonstrated that their preparation was sensitive to calcium but their results exhibited considerable vari-

ability from experiment to experiment. The ATPase of moysin of skeletal and cardiac muscle is stimulated by Ca^{2+} but inhibited by Mg^{2+} (ref. 22). The role of Mg^{2+} in relation to Ca^{2+}-sensitive ATPase is not understood but differs from its role in (Na^+, K^+)-ATPase where Mg^{2+} is essential for activation[1]. In the kidney, we have shown that either Ca^{2+} or Mg^{2+} may activate the enzyme and probably at the same site. Addition of bivalent cation (Mg^{2+} *plus* Ca^{2+}) also produces the same pattern of activation and inhibition. The addition of either cation in concentrations above 5 mM is always inhibitory.

Ca^{2+}-stimulated ATP hydrolysis was always less than that produced by Mg^{2+} but this finding does not necessarily mean that Ca^{2+} in the intact cell would be transported at a lower rate than Mg^{2+}. Indeed, in the red cell where Mg^{2+} ATPase has been found, SCHATZMANN AND VINCENZI[7] in their experiments on Ca^{2+} transport were unable to demonstrate that Mg^{2+} is similarly transported. It would be of great interest to measure binding of Ca^{2+} and Mg^{2+} by our membrane preparation and to correlate it with P_i production. PALMER AND POSEY[24] have done these experiments in rabbit renal cortical membrane preparations and did not find any correlation. They found the rate of Ca^{2+} binding to be greater in the presence of Mg^{2+} than in its absence.

We could not quantitate the relationship between P_i production and the transport of ions, such as has been done by SEN AND POST[23] for Na^+ and K^+ in the red cell, because we used an isolated membrane preparation.

The degree of hydrolysis of ITP, ATP and GTP by the enzyme preparation agrees with that of WHEELER AND WHITTAM[21], and of PALMER AND POSEY[24]. Both groups of investigators demonstrated a similar lack of specificity for ATP. However, PALMER AND POSEY did show that ATP alone could promote Ca^{2+} binding to the membrane preparation whereas other triphosphates did not.

The high activation by Mn^{2+} and not by Sr^{2+} is surprising but in agreement with the results of ROSENTHAL *et al.*[20] on Ca^{2+}-sensitive ATPase in the red cell. The wide plateau of the pH activation curve, the poor substrate specificity, and the activation by Mn^{2+} lead us to suspect that we are not dealing with a single enzyme. Our results, however, show that the hydrolysis of ATP is sensitive to Ca^{2+} and Mg^{2+} and this presumably may have physiological significance. It has been suggested that alkaline or acid phosphatase may be present in the preparation but the pH activation curve does not support this idea.

In our preliminary experiments, we demonstrated that 70 mM Tris reduced P_i production at low Ca^{2+} concentrations (1 mM) but were unable to explain this. Attempts to measure differences in Ca^{2+} ion activity in 70 mM and 20 mM Tris using a Ca^{2+} selective electrode failed to show any appreciable influence of Tris on Ca^{2+} activity.

Ca^{2+} and Mg^{2+} must both be transported by the renal tubule and it is suggested that a common transport mechanism for these two divalent ions may exist. Competition between the two ions for this transport mechanism has been reported. In hypercalcemia where there is presumably a large Ca^{2+} load in the tubule, there is concomitant hypermagnesiuria, and the reverse situation is true[25,26]. As we have demonstrated sensitivity to Ca^{2+} or Mg^{2+} with competition between the two ions for activation of ATPase in the kidney cortex plasma membrane, we believe that this enzyme plays a role in the transport of Ca^{2+} and Mg^{2+} by the kidney.

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