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# CALCIUM INHIBITION OF THE ATPase AND PHOSPHATASE ACTIVITIES OF $(Na^+ + K^+)$-ATPase

LUIS BEAUGÉ and MARTA A. CAMPOS

*División de Biofísica, Instituto de Investigación Médica Mercedes y Martin Ferreyra, Casilla de Correo 389, 5000 Córdoba (Argentina)*

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**In experiments performed at 37°C, $Ca^{2+}$ reversibly inhibits the $Na^+$- and $(Na^+ + K^+)$-ATPase activities and the $K^+$-dependent phosphatase activity of $(Na^+ + K^+)$-ATPase. With 3 mM ATP, the $Na^+$-ATPase was less sensitive to $CaCl_2$ than the $(Na^+ + K^+)$-ATPase activity. With 0.02 mM ATP, the $Na^+$-ATPase and the $(Na^+ + K^+)$-ATPase activities were similarly inhibited by $CaCl_2$. The $K_{0.5}$ for $Ca^{2+}$ as $(Na^+ + K^+)$-ATPase inhibitor depended on the total $MgCl_2$ and ATP concentrations. This $Ca^{2+}$ inhibition could be a consequence of $Ca^{2+}$-$Mg^{2+}$ competition, Ca·ATP-Mg·ATP competition or a combination of both mechanisms. In the presence of $Na^+$ and $Mg^{2+}$, $Ca^{2+}$ inhibited the $K^+$-dependent dephosphorylation of the phosphoenzyme formed from ATP, had no effect on the dephosphorylation in the absence of $K^+$ and inhibited the rephosphorylation of the enzyme. In addition, the steady-state levels of phosphoenzyme were reduced in the presence both of NaCl and of NaCl plus KCl. With 3 mM ATP, $Ca^{2+}$ alone sustained no more than 2% of the $(Na^+ + K^+)$-ATPase activity and about 23% of the $Na^+$-ATPase activity observed with $Mg^{2+}$ and no $Ca^{2+}$. With 0.003 mM ATP, $Ca^{2+}$ was able to maintain about 40% of the $(Na^+ + K^+)$-ATPase activity and 27% of the $Na^+$-ATPase activity seen in the presence of $Mg^{2+}$ alone. However, the $E_2(K)$-$E_1K$ conformational change did not seem to be affected. $Ca^{2+}$ inhibition of the $K^+$-dependent *p*-nitrophenylphosphatase activity of the $(Na^+ + K^+)$-ATPase followed competition kinetics between $Ca^{2+}$ and $Mg^{2+}$. In the presence of 10 mM NaCl and 0.75 mM KCl, the fractional inhibition of the $K^+$-dependent *p*-nitrophenylphosphatase activity as a function of $Ca^{2+}$ concentration was the same with and without ATP, suggesting that $Ca^{2+}$ indeed plays the important role in this process. In the absence of $Mg^{2+}$, $Ca^{2+}$ was unable to sustain any detectable ouabain-sensitive phosphatase activity, either with *p*-nitrophenylphosphate or with acetyl phosphate as substrate.**

## Introduction

Most mammalian cells maintain an asymetric $Na^+$ and $K^+$ distribution across their plasma membrane on the basis of an active $Na^+$-$K^+$ exchange mechanism (see Ref. 1 for references). This system sustains a relatively low $Na_i^+$ and high $K_i^+$ concentration by expelling $Na^+$ from, and taking $K^+$ into, the cells. The required energy is supplied by the hydrolysis of ATP [1,2]. The coupling of this chemiosmotic work is performed by the membrane-bound $(Na^+ + K^+)$-ATPase enzyme, which is likely to be an integral part of the cation-translocating mechanism [1,2]. A divalent physiological cation, $Mg^{2+}$, is essential in the activation of the $(Na^+ + K^+)$-ATPase [2], whereas another divalent physiological cation, $Ca^{2+}$, acts as an inhibitor of that activity [3–9]. The effects of the two aforementioned cations are intracellular [1,2]. Two questions are basic to the effects of $Ca^{2+}$ on $(Na^+$

$+ K^+$)-ATPase; one is related to its possible physiological role and the second to its use as a tool in understanding the mechanism of $Na^+,K^+$-dependent ATP hydrolysis. Regarding the first, it is now accepted that in non-excitable cells as well as in excitable cells at rest (muscle, nerve, secretory cells) the intracellular $Ca^{2+}$ concentration is about or below 0.1 $\mu$M [10]; at this $Ca_i^{2+}$ concentration, the $Na^+$-$K^+$ transport system seems to work reasonably well. However, in excitable cells during activity the $Ca_i^{2+}$ concentration, especially in regions near the cell membrane, can reach levels of 10 $\mu$M or more [10], and at these concentrations some effects on the $Na^+$-$K^+$ transport cannot be ruled out. The second question has to do with the mechanisms of $Ca^{2+}$ inhibition. It seems established that $Ca^{2+}$ inhibits the ($Na^+ + K^+$)-ATPase by acting at more than one step in the hydrolytic cycle [7–9]. Some of them are well documented, but the nature of the inhibition as well as the relative importance of these individual steps on the overall inhibitory effect of $Ca^{2+}$ are not clear. The reason for this is that different experiments were performed under quite different conditions; for instance, whereas the ATP hydrolysis was measured at 37°C, phosphorylation experiments were carried out at 0°C and at different concentrations of ATP [7–9]. The aims of the present work are (i) to establish whether $Ca^{2+}$ at the concentrations likely to be found in excitable cells during activity could have some effect on the $Na^+$-$K^+$ active transport system, (ii) to evaluate the effect of $Ca^{2+}$ on the steps of the ATP hydrolysis under different experimental conditions, (iii) to use the phosphatase activity of the ($Na^+ + K^+$)-ATPase as an additional tool to study the nature of $Ca^{2+}$ inhibition.

## Methods

The experiments were performed on partially purified pig kidney ($Na^+ + K^+$)-ATPase enzyme obtained by the method of Jørgensen [11]; the usual purity was 10–12 units/mg protein. The preparation, stored at 0°C, lost about 10% of its activity per month. ATPase activity was determined in three ways: (a) by a modification of the method of Fiske and SubbaRow [12] using the Amidol reagent [13]; (b) determining the release of $^{32}P_i$ from ($\gamma$-$^{32}$P)-labelled ATP by extraction with isobutanol/benzene; the labelling of the [$^{32}$P]ATP was performed according to Glynn and Chappell [14] as modified by De Meis [15]; (c) following the hydrolysis of ATP through the oxidation of NADH at 340 nm using a 552 Perkin-Elmer spectrophotometer with a thermostatically controlled cell holder equipped with a magnetic stirrer; these assays were done in the presence of 0.26 mM NADH, 1.4 mM phospho*enol*pyruvate, 10 $\mu$g/ml pyruvate kinase, 10 $\mu$g/ml lactate dehydrogenase and 1 mM dithiothreitol. Phosphatase activity was measured using *p*-nitrophenyl phosphate or acetyl phosphate as a substrate. With *p*-nitrophenyl phosphate the reaction was stopped with 3 vol. of a solution containing 2.5% SDS, 4 mM EDTA and 0.2 M NaOH [16] and the *p*-nitrophenol produced was estimated by the absorbance at 410 nm. In some cases, the production of *p*-nitrophenol was followed in the same incubation media into a thermostatically controlled cell using a 552 Perkin-Elmer spectrophotometer and reading the absorbance at 410 nm (pH 7.4). The acetyl phosphatase activity was assayed according to Israel and Titus [17] following the disappearance of acetyl phosphate by the hydroxylamine method of Stadtman [18]. In all experiments, total substrate hydrolysis never exceeded 10%. Steady-state phosphorylation of the protein from ATP was done as in Ref. 13. Pre-steady-state phosphorylation was performed in a rapid mixing apparatus as described in Ref. 19. The concentration of the enzyme was 50 $\mu$g/ml and the temperature 16°C. The reaction was stopped in the same volume of an ice-cold solution containing 5 mM ATP, 50 mM phosphoric acid and 20% (w/v) perchloric acid. After standing in the cold for 15 min the protein precipitated was collected on Whatman FG/C glass-fibre filters and washed with 40 ml ice-cold 5% trichloroacetic acid/50 mM phosphoric acid. The filters were then deposited in counting vials and counted after the addition of 5 ml scintillator. We are grateful to P.J. Garrahan and A. Rega for allowing us to use their rapid mixing equipment and their facilities. Protein was determined by a modification [20] of the method of Lowry et al. [21].

All solutions were made with bidistilled deionized water. NaCl and KCl were Ultrex Grade

from Baker; all other chemicals were reagent grade. ATP, *p*-nitrophenyl phosphate (di-Tris salt), acetyl phosphate and ouabain were obtained from Sigma. Vanadium-free ATP and acetyl phosphate were transformed into their Tris salts after passing through an Amberlite IR-120 cation exchange column. Carrier-free $^{32}P_i$ was purchased from the Comisión Nacional de Energía Atómica of Argentina. As the composition of the solutions varied in the different experiments, it is detailed in each corresponding figure and figure legend.

The calculations of the ionized $Ca^{2+}$ and $Mg^{2+}$ concentrations were done on the basis of the following dissociation constants (mM) taken from Sillén and Martel [22] and corrected for 37°C and pH 7.4: Mg · ATP, 0.091; Mg · EGTA, 5.5; Mg · *p*-nitrophenyl phosphate, 10; Ca · ATP, 0.165; Ca · EGTA, 0.000031; Ca · *p*-nitrophenyl phosphate, 20. The dissociation constants for Mg · acetyl phosphate and Ca · acetyl phosphate were arbitrarily taken equal to those for *p*-nitrophenyl phosphate.

Radioactivity was essayed in a Beckman liquid scintillation counter using a Triton X-100-toluene based scintillator. Where possible, counting was long enough to allow a standard error of 1%. Unless otherwise stated, all experiments were carried out in triplicate; each experiment was repeated at least once.

## Results

### *Calcium inhibition of the $Na^+$- and ($Na^+ + K^+$)-ATPase activities*

In preliminary experiments (not shown) we observed that in the presence of 3 mM $MgCl_2$ and 3 mM ATP, 1 mM $CaCl_2$ produced a large inhibition of the $Na^+,K^+$-dependent ATPase activity. Similar results were seen when the enzyme was preincubated (15 min at 37°C) in the absence of ATP (and the reaction started by adding the nucleotide) or when the reaction begun with the addition of the enzyme to the ATP-containing solutions. On the other hand, inhibition could be prevented, or fully reversed, by including enough EGTA to chelate the $Ca^{2+}$ present in the media.

After those initial observations, we decided to explore the effects of different $CaCl_2$ concentrations on the $Na^+$- and the ($Na^+ + K^+$)-ATPase activities at high and low levels of ATP, that is, under conditions in which different steps in the reaction are rate limiting [1,2]. Thus, in the presence of $Na^+$ and $K^+$, high ATP concentrations are required in order to accelerate the release of $K^+$ from the enzyme after this cation has promoted dephosphorylation. On the other hand, in the presence of $Na^+$ alone, ATP concentrations above those needed to saturate the catalytic site are irrelevant, for the occluded $K^+$-enzyme complex is not formed under these conditions. The experiments are summarized in Fig. 1, where the percentage of inhibition of the ouabain-sensitive ATP hydrolysis is plotted against the total $CaCl_2$ concentration. With 3 mM ATP, the $Na^+,K^+$-dependent hydrolysis was far more sensitive to $CaCl_2$ than the hydrolysis in the presence of $Na^+$ alone. On the other hand, with ATP in the micromolar range the $Na^+$- and the $Na^+,K^+$-dependent hydrolyses were more resistant to, and similarly affected by, $CaCl_2$. Actually, all points at 0.02 mM ATP and the $Na^+$-dependent points at 3 mM ATP fell

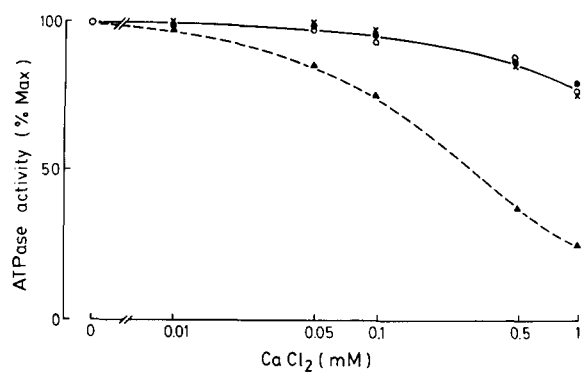


Fig. 1. Effect of different $CaCl_2$ concentrations on the $Na^+$- and ($Na^+ + K^+$)-ATPase activities of purified ($Na^+ + K^+$)-ATPase in the presence of 3 mM or 0.02 mM ATP. The composition of the incubation solutions was as follows (mM): (a) $Na^+$-ATPase: NaCl, 130; Tris-HCl, pH (37°C) 7.4, 50; $MgCl_2$, 3; EGTA, 0.1; (b) ($Na^+ + K^+$)-ATPase: NaCl, 130; KCl, 20; Tris-HCl, pH (37°C) 7.4, 30; $MgCl_2$, 3; EGTA, 0.1. Each point is the mean of triplicate determinations. The ($Na^+ + K^+$)-ATPase and the $Na^+$-ATPase activities were taken as the difference between the ATP hydrolysis found in the absence and presence of $10^{-3}$ M ouabain. ○, $Na^+$-ATPase at 0.02 mM ATP; ●, $Na^+$-ATPase at 3 mM ATP; ×, ($Na^+ + K^+$)-ATPase at 0.02 mM ATP; ▲, ($Na^+ + K^+$)-ATPase at 3 mM ATP. Note that the $CaCl_2$ concentrations given in the figure are the differences between the total $CaCl_2$ and the EGTA concentrations.

on the same line. However, if ionized calcium concentration is what matters, it must be noticed that $Ca^{2+}$ will be the same for the $Na^+$- and the $Na^+$,$K^+$-dependent activities at a given ATP concentration, but will be higher at low than at high ATP. This would mean that at low ATP both the $Na^+$- and $Na^+$,$K^+$-dependent ATP hydrolysis are more resistant to $Ca^{2+}$ than the $(Na^+ + K^+)$-ATPase activity at high ATP concentration. Incidentally, the $K_{0.5}$ for $CaCl_2$ in the inhibition of the $(Na^+ + K^+)$-ATPase activity in Fig. 2 is practically the same as that reported by Tobin et al. [7] for rat brain enzyme under similar conditions. It should be also mentioned at this point that the $CaCl_2$ sensitivity of the $(Na^+ + K^+)$-ATPase activity was the same with 20 mM KCl/130 mM NaCl and with 135 mM KCl/15 mM NaCl (not shown); the latter ionic conditions are those to be expected for the internal environment in a mammalian cell.

The effects of the $Ca^{2+}$ on the $(Na^+ + K^+)$-ATPase activity were explored at different $MgCl_2$ and ATP concentrations and the data were normalized expressing inhibition as a percentage of the activity in the absence of $Ca^{2+}$. Fig. 2 shows a family of curves with the $K_{0.5}$ for $Ca^{2+}$ going from about 10 $\mu$M up to about 600 $\mu$M. The $(Na^+ + K^+)$-ATPase activity appears more resistant to $Ca^{2+}$ when $MgCl_2$ exceeds ATP, and more sensitive if the reverse is the case, suggesting that inhibition of the $(Na^+ + K^+)$-ATPase by $Ca^{2+}$ follows a complex relationship with $Ca^{2+}$, $MgCl_2$ and ATP. The percentage inhibition was also plotted as a function of the Ca-ATP complex concentration (not shown); this plot also showed a family of curves, indicating that calcium effects on the $(Na^+ + K^+)$-ATPase activity are not simply related to the concentration of the Ca-ATP complex.

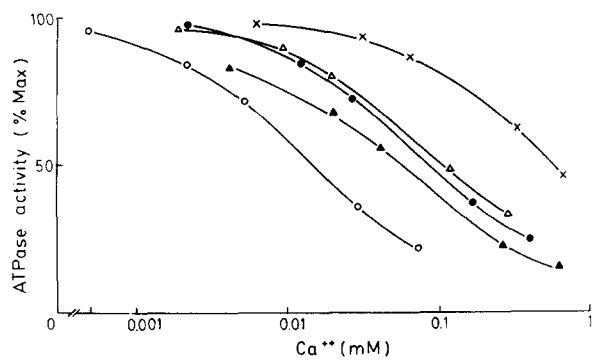


Fig. 2. $(Na^+ + K^+)$-ATPase activity in purified $(Na^+ + K^+)$-ATPase as a function of the ionized $Ca^{2+}$ concentrations in the presence of different ATP and $MgCl_2$ concentrations. The composition of the incubation solutions was as follows (mM): NaCl, 130; KCl, 20; Tris-HCl, pH (37°C) 7.4, 30. Each point is the mean of triplicate determinations. Temperature was 37°C. ●, 3 mM ATP/3 mM $MgCl_2$; ○, 6 mM ATP/3 mM $MgCl_2$; ×, 3 mM ATP/6 mM $MgCl_2$; △, 6 mM ATP/6 mM $MgCl_2$; ▲, 1 mM ATP/1 mM $MgCl_2$. The $Ca^{2+}$ concentrations were calculated on the basis of the dissociation constant given in Methods.

If one assumes that calcium inhibition develops because ionized calcium competes with ionized magnesium for some enzyme site, the following expression can be obtained

$$\frac{1}{\%} = 0.01 + 0.01 \cdot \frac{K_{Mg}}{K_{Ca}} \cdot \frac{[Ca^{2+}]}{[Mg^{2+}]} \qquad (1)$$

where % is the percentage of the ATPase activity in the absence of $Ca^{2+}$; $K_{Mg}$ and $K_{Ca}$ are the dissociation constants of the enzyme-Mg and enzyme-Ca complex, respectively; $[Ca^{2+}]$ and $[Mg^{2+}]$ are the $Ca^{2+}$ and $Mg^{2+}$ concentrations. Thus, for a $Ca^{2+}$-$Mg^{2+}$ competition the equation predicts a straight-line relationship between the reciprocal of the ATPase activity (taken as a percentage of that found in the absence of $Ca^{2+}$) and the $Ca^{2+}/Mg^{2+}$ concentration ratio. Fig. 3 is actually a plot of this type taking several points corresponding to the experiments described in Fig. 2 for different $MgCl_2$ and ATP concentrations; as is clearly seen, all points fall reasonably well on a single straight line, regardless of the $MgCl_2$, ATP or $CaCl_2$ levels. The values illustrated in Fig. 3 are those which allow one to observe in detail the intercepts on the vertical and horizontal axes, but all points from the data fell on a single straight line. The line in Fig. 3 intercepts the vertical axis at the expected value, 0.01 (100% ATPase activity in the absence of $Ca^{2+}$). From the intercepts on the horizontal axis, the ratio between the dissociation constants of $Ca^{2+}$ and $Mg^{2+}$ with the hypothetical enzyme site can be obtained; the ratio is about 0.16, indicating that the affinity of that site for $Ca^{2+}$ is about 6-times higher than for $Mg^{2+}$.

It must be stressed here that other mechanisms will equally well satisfy the results of Figs. 2 and 3. This comes from the fact that $Ca^{2+}$ and $Mg^{2+}$

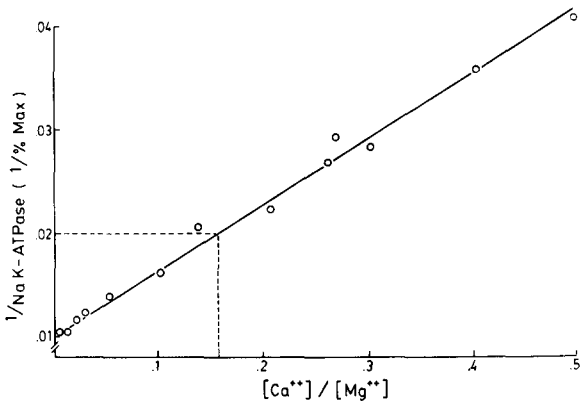


Fig. 3. Replot of some of the data from Fig. 2 taking the reciprocal of the $(Na^+ + K^+)$-ATPase activity (as percentage of that found in the absence of $Ca^{2+}$) as a function of the $Ca^{2+}/Mg^{2+}$ ratio. The $Ca^{2+}$ and $Mg^{2+}$ concentrations were calculated in accordance with the dissociation constants given in Methods.

concentrations will relate to the concentrations of the Ca-ATP and Mg-ATP complexes through their respective dissociation constants in a multiple equilibrium. Considering that $Ca^{2+}$ inhibition represents competition between Ca · ATP and Mg · ATP, an equation similar to Eqn. 1 can be derived. This is

$$\frac{1}{\%} = 0.01 + 0.01 \cdot \frac{K_{Mg \cdot ATP}}{K_{Ca \cdot ATP}} \cdot \frac{[Ca \cdot ATP]}{[Mg \cdot ATP]} \quad (2)$$

where: $K_{Mg \cdot ATP}$ and $K_{Ca \cdot ATP}$ are the dissociation constants related to the Ca · ATP-Mg · ATP enzyme binding site; [Ca · ATP] and [Mg · ATP] are the concentrations of the ATP-divalent cation complexes; and the rest is the same as in Eqn. 1. The plot of the data from Fig. 2 as a function of the CA · ATP/Mg · ATP ratio also falls into a straight line as predicted by Eqn. 2 (not shown). From the horizontal intercept, the $K_{Ca \cdot ATP}/K_{Mg \cdot ATP}$ ratio is 0.085; that is, if $Ca^{2+}$ inhibits the $(Na^+ + K^+)$-ATPase activity because Ca · ATP competes with Mg · ATP the affinity of the enzyme site for Ca · ATP is about 12-times higher than the affinity for the Mg-ATP complex.

In another series of experiments we investigated the $Na^+$ and $K^+$ activation curves of the $(Na^+ + K^+)$-ATPase in the absence and presence of calcium. This was motivated in part by the report of Tobin et al. [7] that calcium inhibited phosphorylation of the enzyme by competing with $Na^+$ and not with $Mg^{2+}$, contradicting our data of Fig. 3 and our observation that similar inhibition

TABLE I

APPARENT AFFINITIES, TAKEN AS THE $K_{0.5}$ VALUES, FOR NaCl AND KCl IN THE ACTIVATION OF THE $(Na^+ + K^+)$-ATPase IN THE ABSENCE AND PRESENCE OF $CaCl_2$

The composition of the incubation solutions was the following (mM): ATP, 3; $MgCl_2$, 3; EGTA, 0.1; Tris-HCl, pH (37°C) 7.4, 180 − (NaCl + KCl). When present, $CaCl_2$ was at 0.6 mM; at this concentration the inhibition averaged 50%. For the KCl activation curves, NaCl was kept constant at 130 mM and KCl was varied between zero and 20 mM. For the NaCl activation curves KCl was maintained constant at 20 mM, whereas NaCl was varied from zero to 130 mM.

| | NaCl | | | | KCl | | | |
|---|---|---|---|---|---|---|---|---|
| | $-Ca^{2+}$ | $+Ca^{2+}$ | $\Delta Ca^{2+}$ | | $-Ca^{2+}$ | $+Ca^{2+}$ | $\Delta Ca^{2+}$ | |
| | (mM) | (mM) | mM | % | (mM) | (mM) | mM | % |
| | 14 | 14 | 0 | 0 | 1.3 | 0.8 | −0.5 | −38 |
| | 14 | 15 | +1 | +7 | 1.1 | 0.7 | −0.4 | −36 |
| | 11 | 12 | +1 | +9 | 1.3 | 0.7 | −0.6 | −46 |
| Mean | 13 | 13.7 | +0.67 [a] | +5.3 [a] | 1.23 | 0.73 | −0.50 [b] | −40 [b] |
| S.E. | | | ±0.33 | ±2.7 | | | ±0.06 | ±3 |

[a] Difference from zero not significant.
[b] Difference from zero $P < 0.001$.

curves by $CaCl_2$ were obtained with 130 mM NaCl/20 mM KCl and with 15 mM NaCl/135 mM KCl. We took as the expression of the apparent affinity for the tested cation, the $K_{0.5}$ value in the activation curve. The results, summarized in Table I, indicate that $CaCl_2$, at concentrations giving about 50% reduction in the $(Na^+ + K^+)$-ATPase activity (i) did not alter the apparent affinity for $Na^+$, and (ii) produced a significant increase in the apparent affinity for $K^+$.

*The effects of calcium on the levels of phosphoenzyme*

In order to analyze the relative importance of $Ca^{2+}$ in the intermediary steps of ATP hydrolysis, we started by studying its effects on steady-state levels of phosphoenzyme at 37°C (Table II). In the absence of $CaCl_2$ and $K^+$, levels of phosphoenzyme were the same with 1 mM and 0.02 mM ATP; on the other hand, 20 mM KCl largely reduced the phosphoenzyme values, this being more pronounced at 0.02 mM ATP ($P < 0.001$). Our results reproduce those of Post et al. [23] at 0°C, in agreement with the accepted 'regulatory' role of ATP in promoting rephosphorylation of the enzyme. It is important to notice that with 1 mM ATP, the amount of phosphoenzyme present during $Na^+$,$K^+$-stimulated enzyme turnover at 37°C is not negligible, for it represents about 23% of that found in the presence of $Na^+$ alone. The addition of 1 mM $CaCl_2$ reduced the steady-state levels of phosphoenzyme under all experimental conditions tested. In the absence of KCl, the decrement was of the order of 23% with 1 mM and 0.02 mM ATP. In the presence of 20 mM KCl, the action of $Ca^{2+}$ was more marked with 1 mM ATP (65% reduction in phosphoenzyme) than with 0.02 mM ATP (45% reduction). If one looks at the ATP concentration effects in the $Ca^{2+}$-inhibited enzyme, it is seen that they reproduce qualitatively the findings in the absence of $Ca^{2+}$; quantitatively there is a difference in the sense that with 1 mM ATP the steady-state phosphoenzyme value in 20 mM KCl was only 10% of that found when potassium was excluded.

We see, then, a reduction in the levels of phosphoenzyme produced by $CaCl_2$ concentrations which inhibit the ATPase activity. This could be because (i) $Ca^{2+}$ inhibits only the rephosphorylating part of the cycle, or (ii) there is an inhibition of dephosphorylation, but this is accompanied by an even larger inhibition of the rephosphorylation

TABLE II

EFFECTS OF $Ca^{2+}$ IN THE PRESENCE OF $Mg^{2+}$ ON THE $Na^+$- AND $(Na^+ + K^+)$-ATPase ACTIVITIES, STEADY-STATE LEVELS OF PHOSPHOENZYME AND ESTIMATED RATES OF DEPHOSPHORYLATION IN PURIFIED $(Na^+ + K^+)$-ATPase INCUBATED AT 37°C

All listed cations were as their chloride salts. The incubation solutions had, in addition, 30 mM Tris-HCl, pH (37°C) 7.4, (or 50 mM (K-free)) and 0.1 mM EGTA. Temperature was 37°C. Phosphorylation time was 10 s. In all cases, the phosphorylation blank consisted of the $^{32}P_i$ incorporated into the protein previously denatured with perchloric acid. ATPase activities were determined following the release of $^{32}P_i$ from ($\gamma$-$^{32}P$)-labelled ATP. The rate constant for dephosphorylation ($k$) were calculated on the basis that ATPase activity = $k \cdot$[EP]. Note that in the absence of $K^+$ the rate of dephosphorylation was practically not affected by $Ca^{2+}$, whereas in the presence of 20 mM $K^+$ the dephosphorylation was about 50% inhibited.

| Concentrations (mM) | | | | | ATPase ($nmol \cdot mg^{-1} \cdot s^{-1}$) | Phosphoenzyme ($nmol \cdot mg^{-1}$) | $k$ ($s^{-1}$) |
|---|---|---|---|---|---|---|---|
| $Na^+$ | $K^+$ | $Mg^{2+}$ | $Ca^{2+}$ | ATP | | | |
| 130 | – | 3 | – | 1 | 4.7 ± 0.1 | 2.41 ± 0.1 | 1.95 |
| 130 | – | 3 | 1.1 | 1 | 3.5 ± 0.1 | 1.91 ± 0.1 | 1.83 |
| 130 | 20 | 3 | – | 1 | 114.0 ± 4.0 | 0.58 ± 0.03 | 197 |
| 130 | 20 | 3 | 1.1 | 1 | 19.0 ± 0.8 | 0.19 ± 0.03 | 100 |
| 130 | – | 3 | – | 0.02 | – | 2.62 ± 0.12 | – |
| 130 | – | 3 | 1.1 | 0.02 | – | 2.00 ± 0.10 | – |
| 130 | 20 | 3 | – | 0.02 | – | 0.15 ± 0.02 | – |
| 130 | 20 | 3 | 1.1 | 0.02 | – | 0.08 ± 0.01 | – |

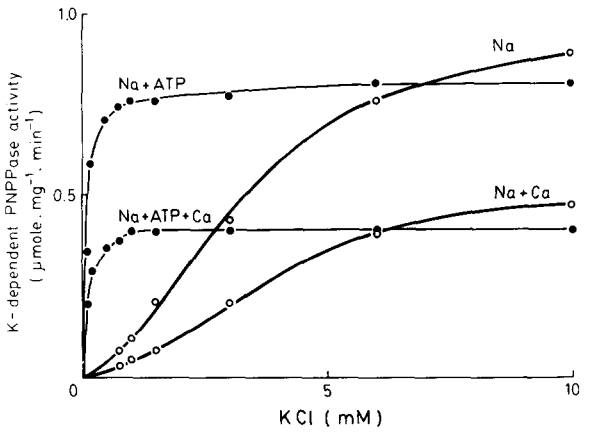


Fig. 4. Effects of $Ca^{2+}$ on the activation by $K^+$ of *p*-nitrophenyl phosphate hydrolysis in purified ($Na^+ + K^+$)-ATPase incubated in the presence of NaCl alone or in NaCl plus ATP. The composition of the solutions was as follows (mM): Tris-HCl, pH (37°C) 7.4, 150 minus KCl; NaCl, 10; *p*-nitrophenyl phosphate, 5; $MgCl_2$, 3; EGTA, 0.1; KCl, variable; ATP, zero or 0.1; $CaCl_2$ zero or 0.6. Each point is the mean of triplicate determinations. See Methods for details.

step. The fact that we actually wanted to work as close as possible to physiological conditions, and chose 37°C as the working temperature, left us with no real chance to investigate directly the effects of $Ca^{2+}$ on dephosphorylation. However, we did that indirectly, based on the simplified view that ATPase activity can be expressed by a product of a rate constant and the amount of phosphoenzyme present. We determined the $Na^+$- and ($Na^+ + K^+$)-ATPase activities under the same experimental conditions as those at which the phosphoenzyme levels were determined (at 1 mM ATP). From the known rates of ATP hydrolysis and phosphoenzyme levels, an estimate of the rate constant for dephosphorylation was obtained. These results, collected in Table II, indicate that (i) in the absence of K, $CaCl_2$ had little or no effect on the rate of dephosphorylation, and (ii) in the presence of 20 mM KCl, 1 mM $CaCl_2$ produced a 50% inhibition in the dephosphorylation rate. The calculated rate constant for dephosphorylation of 197 $s^{-1}$ in the absence of $CaCl_2$ corresponds to a turnover number of about 11 800 $min^{-1}$, which although on the high side, is not unreasonable for this enzyme system [24]; this in turn suggests that the estimates given above are not unrealistic. Taken altogether, the results show that for physiological ATP and $Mg^{2+}$ concentrations, and at 37°C, $Ca^{2+}$ inhibition of the ($Na^+ + K^+$)-ATPase activity is exerted in two ways: (1) a reduction in the rate of dephosphorylation of the phosphoenzyme; and (2) an even more pronounced inhibition of the enzyme rephosphorylation. In the case of the $Na^+$-ATPase activity, inhibition by $Ca^{2+}$ can be explained fully by the reduction in the rephosphorylation step.

*$Ca^{2+}$ and the $K^+$-disoccluding step*

The experiments that follow were designed to evaluate indirectly the possibility that $Ca^{2+}$ might affect $K^+$ disocclusion. We compared first the ability of $Ca^{2+}$ with that of $Mg^{2+}$ in sustaining an ATPase activity under conditions where the $E_2$-$E_1$ conformation step had different relevance to the overall ATPase activity. These experiments, summarized in Table III, show the following: (i) in the presence of 3 mM ATP, $Ca^{2+}$ was able to sustain only 2% of the ($Na^+ + K^+$)-ATPase activity but 23% of the $Na^+$-ATPase found with similar concentrations of $Mg^{2+}$ (see also Ref. 9); (ii) with 0.003 mM ATP, $Ca^{2+}$ could sustain as much as 41% of the ($Na^+ + K^+$)-ATPase activity and 27% of the $Na^+$-ATPase activity observed in the presence of $Mg^{2+}$; (iii) there was no difference between the $Na^+$-dependent ATP hydrolysis at 3 mM ATP and at 0.003 mM ATP – this was seen both with $Mg^{2+}$ and $Ca^{2+}$ in the incubation solutions; (iv) ATP stimulated the $Na^+$,$K^+$-dependent ATP hydrolysis in $Mg^{2+}$- and also in $Ca^{2+}$-containing media; however, the stimulation was much higher in the case of $Mg^{2+}$ (100-fold) than in that of $Ca^{2+}$ (6-fold).

In a second group of experiments (Table IV) we studied the effects of adding $Ca^{2+}$ to $Mg^{2+}$, or replacing $Mg^{2+}$ by $Ca^{2+}$, on ouabain-sensitive ATP hydrolysis in the presence of 3 mM ATP and of $K^+$ or some of its congeners (the $Na^+$-ATPase activity is included as a reference). The rationale was that if the results of Table III at high ATP represent the inability of Ca · ATP to accelerate the $E_2(K)$-$E_1K$ conformational change, one could expect the rates of ATP hydrolysis with $Ca^{2+}$ to be higher with those cations forming a looser complex with the enzyme after dephosphorylation (the stability of the complexes follows the sequence Rb >

TABLE III

EFFECTS OF REPLACING $Mg^{2+}$ BY $Ca^{2+}$ ON THE OUABAIN-SENSITIVE ATP HYDROLYSIS ACTIVATED BY $Na^+$ AND BY $Na^+ + K^+$ IN THE PRESENCE OF 3 mM AND OF 0.003 mM ATP

In addition to $MgCl_2$, $CaCl_2$ and ATP, the incubation solutions had the following composition (mM): (a) ($Na^+ + K^+$)-ATPase: NaCl, 130; KCl, 20; Tris-HCl, pH (37°C) 7.4, 30; EGTA, 0.1; (b) $Na^+$-ATPase: NaCl, 130; Tris-HCl, pH (37°C) 7.4, 50; EGTA, 0.1. The ATPase activities were determined in the absence and presence of $1 \cdot 10^{-3}$ M ouabain by the release of $^{32}P_i$ from [$\gamma$-$^{32}$P]ATP. The ionized $Ca^{2+}$ and $Mg^{2+}$ concentrations were calculated on the basis of the dissociation constant given in Methods. See text for details. Each value is the mean ± S.E. of triplicate determinations.

| Concentration (mM) | | | ATPase | Activity | |
|---|---|---|---|---|---|
| ATP | $Mg^{2+}$ | $Ca^{2+}$ | | $\mu mol \cdot mg^{-1} \cdot min^{-1}$ | % |
| 3 | 0.50 | – | $Na^+ + K^+$ | 11.000 ± 0.090 | 100 |
| 3 | – | 0.60 | $Na^+ + K^+$ | 0.260 ± 0.01 | 2 |
| 3 | 0.50 | – | $Na^+$ | 0.392 ± 0.008 | 100 |
| 3 | – | 0.60 | $Na^+$ | 0.092 ± 0.008 | 23 |
| 0.003 | 0.50 | – | $Na^+ + K^+$ | 0.109 ± 0.008 | 100 |
| 0.003 | – | 0.60 | $Na^+ + K^+$ | 0.045 ± 0.003 | 41 |
| 0.003 | 0.50 | – | $Na^+$ | 0.410 ± 0.01 | 100 |
| 0.003 | – | 0.60 | $Na^+$ | 0.111 ± 0.003 | 27 |

TABLE IV

EFFECTS OF ADDING $Ca^{2+}$ TO $Mg^{2+}$ OR REPLACING $Mg^{2+}$ BY $Ca^{2+}$ ON OUABAIN-SENSITIVE ATP HYDROLYSIS ACTIVATED BY DIFFERENT MONOVALENT CATIONS IN THE PRESENCE OF 3 mM ATP

Mono and divalent cations were present as chloride salts. The buffer used was Tris-HCl, pH (37°C) 7.4, at 30 mM concentration. LiCl was at 90 mM in order to have it at saturating concentrations, thus avoiding any bias due to eventual changes in the apparent affinity for the activating cations. In all cases, there was 0.1 mM EGTA. Each value is the mean ± S.E. of triplicate determinations.

| Concentration (mM) | | | | | | | Ouabain-sensitive ATPase activity | |
|---|---|---|---|---|---|---|---|---|
| $Na^+$ | $K^+$ | $Rb^+$ | $NH_4^+$ | $Li^+$ | $Mg^{2+}$ | $Ca^{2+}$ | $\mu mol\ P_i \cdot mg^{-1} \cdot min^{-1}$ | % |
| 120 | 30 | – | – | – | 3 | – | 10.80 ± 0.12 | 100 |
| 120 | 30 | – | – | – | 3 | 1.1 | 2.52 ± 0.03 | 23 |
| 120 | 30 | – | – | – | – | 3.1 | 0.250 ± 0.01 | 2.3 |
| 120 | – | 30 | – | – | 3 | – | 9.80 ± 0.12 | 100 |
| 120 | – | 30 | – | – | 3 | 1.1 | 2.22 ± 0.04 | 23 |
| 120 | – | 30 | – | – | – | 3.1 | 0.259 ± 0.01 | 2.6 |
| 120 | – | – | 30 | – | 3 | – | 10.70 ± 0.04 | 100 |
| 120 | – | – | 30 | – | 3 | 1.1 | 2.47 ± 0.03 | 23 |
| 120 | – | – | 30 | – | – | 3.1 | 0.290 ± 0.02 | 2.7 |
| 60 | – | – | – | 90 | 3 | – | 3.29 ± 0.02 | 100 |
| 60 | – | – | – | 90 | 3 | 1.1 | 1.53 ± 0.02 | 47 |
| 60 | – | – | – | 90 | – | 3.1 | 0.270 ± 0.01 | 8 |
| 150 | – | – | – | – | 3 | – | 0.380 ± 0.01 | 100 |
| 150 | – | – | – | – | 3 | 1.1 | 0.280 ± 0.01 | 74 |
| 150 | – | – | – | – | – | 3.1 | 0.105 ± 0.01 | 27 |

$K > NH_4 > Li$) [23]. Actually, the results of Table IV do not indicate any differential effect of $K^+$ and its congeners on the rates of ATP hydrolysis in the presence of $Ca^{2+}$ plus $Mg^{2+}$ or of $Ca^{2+}$ alone. Considering just $K^+$, $Rb^+$ and $NH_4^+$, which give similar maximal velocities at saturating concentrations (and 30 mM is certainly saturating) [25], all produced similar activity either in absolute or relative terms. On the other hand, the larger fraction remaining in $Li^+$ does not seem relevant because (i) it is well known that $Li^+$ does not lead to the same maximal velocity as does $K^+$ [25], and (ii) the absolute rates in $Li^+$ were lower than or the same as with $K^+$ and its congeners. We will come back to these points in the Discussion.

*Effects of $Ca^{2+}$ on pre-steady-state phosphorylation from ATP*

Another way of looking into the rephosphorylation step was to obtain quantitative estimations of the actual rate of transphosphorylation and see if they could account for the observed $Ca^{2+}$ effects. Working at 25°C, Mardh and Post [26] have shown that when the enzyme is premixed with NaCl and then mixed with NaCl, $MgCl_2$ and ATP, phosphorylation as a function of time is not too far from linear up to about 20 ms. We decided then to estimate the transphosphorylation rate by performing a similar experiment, at a lower temperature, taking a single point at 20 ms. The results in Table V show that the addition of 1 mM $CaCl_2$ to 3 mM $MgCl_2$ produced a 42% reduction in phosphoenzyme formation. On the other hand, 3 mM $CaCl_2$ alone gave a phosphorylation value which is only 4% of that obtained with similar concentrations of $MgCl_2$.

TABLE V

EFFECTS OF $Ca^{2+}$ ON PRE-STEADY-STATE PHOSPHORYLATION FROM ATP OF $(Na^+ + K^+)$-ATPase IN THE PRESENCE AND ABSENCE OF $Mg^{2+}$

The enzyme (50 μg protein/ml) was preincubated for 2–3 min in 50 mM Tris-HCl, pH (25°C) 7.4, and 130 mM NaCl. The reaction was started by mixing 1 ml of the enzyme suspension with 1 ml of a solution containing 130 mM NaCl, 50 mM Tris-HCl, pH (25°C) 7.4, 10 μM $^{32}P_i$-ATP and 6 mM $MgCl_2$ or 6 mM $MgCl_2$ + 2 mM $CaCl_2$ or 6 mM $CaCl_2$. After 20 ms at 16°C the reaction was stopped in 2 ml of an $ATP-P_i$/perchloric acid mixture (see Methods). The amount of $P_i$ incorporated into denatured enzyme (enzyme premixed with perchloric acid) was $0.006 \pm 0.0003$ nmol·mg$^{-1}$. Each value is the mean ± S.E. of triplicate determinations.

| Condition | Phosphorylated protein | |
|---|---|---|
| | nmol·mg$^{-1}$ | % |
| 3 mM $MgCl_2$ | $0.474 \pm 0.014$ | 100 |
| 3 mM $MgCl_2$ + 1 mM $CaCl_2$ | $0.277 \pm 0.011$ | 58 |
| 3 mM $CaCl_2$ | $0.019 \pm 0.001$ | 4 |

*Calcium inhibition of the $K^+$-dependent phosphatase activity in purified $(Na^+ + K^+)$-ATPase enzyme*

The $K^+$-dependent phosphatase activity of $(Na^+ + K^+)$-ATPase offers an alternative approach to the study of effects of $Ca^{2+}$ on this enzyme. On the one hand it has the advantage that it can operate in the absence of $Na^+$ and ATP [27,28]; on the other, the fact that it is modified by the presence of $Na^+$ and ATP [23,24] provides the opportunity to study the action of $Ca^{2+}$ with and without the two aforementioned ligands. Acting alone, $Na^+$ and ATP inhibit the $K^+$-dependent *p*-nitrophenylphosphatase activity, reducing the apparent affinity for $K^+$ without changing the maximal rate of hydrolysis; the simultaneous presence of $Na^+$ and ATP increases the apparent affinity for $K^+$ but reduces the maximal activity [27,28]. In the absence of $Na^+$ and ATP, or when either of them is present alone, $K^+$ stimulates *p*-nitrophenyl phosphate hydrolysis acting on intracellular sites, whereas with $Na^+$ plus ATP, $K^+$ is required both intra- and extracellularly [29–31].

Preliminary experiments (not shown) indicated that the phosphatase activity of $(Na^+ + K^+)$-ATPase is reversibly inhibited by $Ca^{2+}$ in a way consistent with a $Ca^{2+}$-$Mg^{2+}$ competition (0.5 mM $CaCl_2$ increased the $K_m$ for $MgCl_2$ from 1.60 to 3.90 mM without chaning the maximal velocity). The plot of the reciprocal of activity as a function of $CaCl_2$ concentration at 2 mM and 8 mM $MgCl_2$ gave an estimated $K_i$ for $CaCl_2$ of about 0.25 mM (5 mM *p*-nitrophenylphosphate). Similar linear plots were obtained when ionized $Ca^{2+}$ and $Mg^{2+}$ concentrations were used instead. Of course, the data do not permit one to rule out a hypothetical Ca *p*-nitrophenyl phosphate–Mg *p*-nitrophenyl

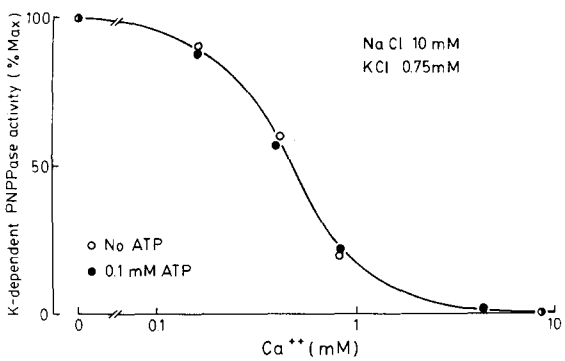


Fig. 5. Effect of different $Ca^{2+}$ concentrations on the $K^+$-dependent *p*-nitrophenylphosphatase (PNPPase) activity in purified $(Na^+ + K^+)$-ATPase. All assays were performed in the presence of NaCl with (●) or without (○) ATP. The data have been normalized given a 100% value to the activities found in the absence of $Ca^{2+}$. The composition of the incubation solutions was the following (mM): Tris-HCl, pH (37°C) 7.4, 150; NaCl, 10; KCl, zero or 0.75; $MgCl_2$, 3; *p*-nitrophenylphosphate, 5; EGTA, 0.1; ATP, zero or 0.1; $CaCl_2$, variable. The $Ca^{2+}$ concentrations were calculated in accordance with the dissociation constant given in Methods. Note that the sensitivity to $Ca^{2+}$ is the same in the presence and absence of ATP. Each value is the mean of triplicate determinations.

phosphate competition as the basis for inhibition.

Calcium inhibition of the phosphatase activity in $(Na^+ + K^+)$-ATPase does not seem to change substantially the characteristics of the potassium activation. This was true in the absence of $Na^+$ and ATP (not shown) as well as in the presence of 10 mM $Na^+$ and of 10 mM $Na^+$ plus 0.1 mM ATP (Fig. 4). Fig. 5 is an experiment complementary to that of Fig. 4, indicating that the apparent affinity for $Ca^{2+}$ as a $K^+$-dependent *p*-nitrophenylphosphatase inhibitor is the same in the case of $Na^+$ and of $Na^+$ plus ATP.

Finally, it is worth noticing that $Ca^{2+}$ could not sustain any ouabain-sensitive phosphatase activity in the absence of $Mg^{2+}$ (Table V). This was seen with high as well as with low concentrations of *p*-nitrophenyl phosphate and also when acetyl phosphate was the substrate.

## Discussion

The work described in this paper was an attempt to characterize the interactions of $Ca^{2+}$ with $(Na^+ + K^+)$-ATPase. Most of the experiments were carried out at 37°C; we used lower temperatures only when absolutely necessary on technical grounds. Our results agree with several reports that have appeared over the years (see Refs. 7 and 9 for references) in the sense that, in the presence of $Mg^{2+}$, $Ca^{2+}$ acts as an inhibitor of the $(Na^+ + K^+)$-ATPase. In addition we have shown that the effects of $Ca^{2+}$ on the ATPase activity are largely dependent on, or influenced by, conditions affecting the extent to which the different intermediary steps in the reaction are rate-limiting.

TABLE VI

EFFECTS OF REPLACING $Mg^{2+}$ BY $Ca^{2+}$ ON THE OUABAIN-SENSITIVE PHOSPHATASE ACTIVITY IN PURIFIED $(Na^+ + K^+)$-ATPase

Divalent cations were present as chloride salts. The other constituents of the solutions were 10 mM KCl, 150 mM Tris-HCl, pH (37°C) 7.4, and 0.1 mM EGTA. The reaction was started with the addition of the substrate after 15 min preincubation at 37°C in the absence and presence of $1 \cdot 10^{-3}$ M ouabain. The actual incubation period lasted 30 min. Each value is the mean ± S.E. of triplicate determinations. The concentrations of $Ca^{2+}$ and $Mg^{2+}$ were calculated on the basis of the dissociation constant given in Methods.

| Substrate | Substrate concentration (mM) | $Mg^{2+}$ (mM) | $Ca^{2+}$ (mM) | Ouabain-sensitive phosphatase activity | |
|---|---|---|---|---|---|
| | | | | $\mu mol \cdot mg^{-1} \cdot min^{-1}$ | % |
| *p*-Nitrophenyl phosphate | 5 | 2.1 | – | 1.160 ± 0.003 | 100 |
| | 5 | – | 2.1 | 0.005 ± 0.003 | 0 |
| | 0.08 | 2.1 | – | 0.037 ± 0.001 | 100 |
| | 0.08 | – | 2.1 | 0.0 ± 0.002 | 0 |
| Acetyl phosphate | 4 | 2.1 | – | 6.7 ± 0.7 | 100 |
| | 4 | – | 2.1 | −0.2 ± 0.4 | 0 |

Our kinetic data on $(Na^+ + K^+)$-ATPase activity indicate that the basis for $Ca^{2+}$ inhibition could be $Ca^{2+}$-$Mg^{2+}$ competition, Ca · ATP-Mg · ATP competition, or both, but does not support the suggestion of Tobin et al. [7] of a $Ca^{2+}$-$Na^+$ antagonism; actually, Fukushima and Nakao [33] produced evidence suggesting that $Na^+$ antagonizes both $Mg^{2+}$ and $Ca^{2+}$ in the transphosphorylation to slow its rate. Two important points emerge from our experiments: (1) $Ca^{2+}$ and $Mg^{2+}$ (or Ca · ATP and Mg · ATP) seem to bind at a single site; and (2) the apparent affinity of the enzyme for $Ca^{2+}$ (and/or for Ca · ATP) is higher than for $Mg^{2+}$ (and/or Mg · ATP). Our data can be also explained if $Ca^{2+}$ and $Mg^{2+}$ (and/or Ca · ATP and Mg · ATP) acting at different places produce two enzyme forms in equilibrium with each other; however, the evidence given by Fukushima and Post [9] and Fukushima and Nakao [33] strongly support the notion that $Ca^{2+}$ indeed binds to the same site as does $Mg^{2+}$.

If $Ca^{2+}$ competes with $Mg^{2+}$, the most likely moment of binding is during transphosphorylation, because $Mg^{2+}$ and $Ca^{2+}$ are already bound to the enzyme in the $E_1$PNa form (Refs. 7,9,33 and results not shown). If what is involved is a Ca · ATP-Mg · ATP competition and $Ca^{2+}$ enters complexed with the nucleotide, there are two possibilities· one is during transphosphorylation; the other is when ATP accelerates the $E_2(K)$-$E_1K$ conformational change. The 'regulatory' role of ATP takes place perfectly well in the absence of $Mg^{2+}$ [32], but this does not mean it cannot function as Mg · ATP. It has been proposed recently [34] that the so-called 'regulatory' ATP site is the same catalytic one undergoing successive changes in affinity; it is conceivable, then, that the ATP-divalent cation complex bound during the dephosphoenzyme transformation can remain attached until, in the presence of $Na^+$, phosphorylation takes place. The implication is that during the normal operational cycle of the $(Na^+ + K^+)$-ATPase activity, $Ca^{2+}$ might get into the enzyme at a step earlier than transphosphorylation.

The considerations we have just gone through open up the possibility that the dephosphoenzyme transformation (disocclusion) is also affected. However, the results in Table IV seem to indicate that this is not the case. There are two ways to reconcile the results of Table IV with a $Ca^{2+}$ inhibition of the dephosphoenzyme transformation: one is that dephosphorylation is so inhibited that it becomes the real rate-limiting step; Table II indicates that this does not happen in the presence of $Ca^{2+}$ plus $Mg^{2+}$, and other experiments (not shown) in the presence of $Ca^{2+}$ alone behaved likewise (see also Refs. 7,33). The other possibility is that the release of $P_i$ and the divalent cation from $E_2$PK is an ordered process in which $P_i$ comes off first; if $Ca^{2+}$ leaves the enzyme more slowly than $Mg^{2+}$ the step

$$CaE_2(K) \rightarrow E_2(K)$$
$$\searrow Ca^{2+}$$

prior to disocclusion might become rate-limiting. Although this hypothesis cannot be completely disproved, the data on pre-steady-state phosphorylation make it unlikely.

In the experiments on pre-steady-state phosphorylation the addition of 1 mM $CaCl_2$ to 3 mM $MgCl_2$ reduced the rate of E-P formation by 40% (Table V); if similar behaviour is observed at 37°C, this reduction, plus the estimated 50% inhibition in the rate of dephosphorylation with $Na^+ + K^+$ (Table II), can account for the inhibition of the $(Na^+ + K^+)$-ATPase activity by $Ca^{2+}$. On the other hand, $Ca^{2+}$ sustains at most 4% of the transphosphorylation rate given by $Mg^{2+}$; this figure can provide the basis for the very low relative $(Na^+ + K^+)$-ATPase activity supported by $Ca^{2+}$ at 3 mM ATP. The large fractional $(Na^+ + K^+)$-ATPase activity in the presence of $Ca^{2+}$ alone at 0.003 mM ATP (Table III) can be explained because the overall cycle of hydrolysis is already slowed down at the disocclusion step. For the $Na^+$-ATPase the rate of dephosphorylation is so slow that even more than 96% inhibition of the rate of transphosphorylation can still sustain more than 20% of ATP hydrolysis. Thus, inhibition of dephosphorylation and transphosphorylation seem enough to account for the effects of $Ca^{2+}$ on the $Na^+$- and $(Na^+ + K^+)$-ATPase activities. The inhibition of the $K^+$-dependent dephosphorylation is probably a consequence of the shift in the $E_1$PNa-$E_2$PNa equilibrium in favour of $E_1$PNa [7,9,33]; a reduction on the forward rate constant of this reaction will reduce the supply of $E_2$PNa, which could

become rate-limiting in the presence of $K^+$ without necessarily affecting the spontaneous (or $Na^+$-stimulated) dephosphorylation. Nevertheless, some slow detachment of $P_i$ from the $Ca^{2+}$-phosphoenzyme cannot be ruled out.

Calcium inhibits the $K^+$-dependent (or ouabain-sensitive) phosphatase activity of the ($Na^+ + K^+$)-ATPase enzyme (Refs. 35,36 and this work) showing competitive behaviour with respect to magnesium. The data are also consistent with both cations binding to a single site, although the reservations between the their identities applies here as well as with the ($Na^+ + K^+$)-ATPase activity. The $K_i$ for $CaCl_2$ (0.25 mM) is 6–7-fold smaller than the $K_m$ for $MgCl_2$ (1.6 mM); if we consider the ionized species, the $K_i$ for $Ca^{2+}$ is 0.2 mM and the $K_m$ for $Mg^{2+}$ is 1 mM. Thus, the ratio between the $Ca^{2+}$ and $Mg^{2+}$ affinities is almost the same for the ($Na^+ + K^+$)-ATPase and the phosphatase activities. This could be just a coincidence, but it could also reflect the fact that the sites at which these cations bind are the same in both instances. There are two main differences between effects of $Ca^{2+}$ on ATPase and phosphatase. On the one hand, in the presence of $Mg^{2+}$, $Ca^{2+}$ inhibition of the $K^+$-dependent phosphatase occurs without changes in the apparent affinity for $K^+$, both in the absence and presence of ATP (Fig. 5). On the other, $Ca^{2+}$ alone is unable to sustain any $K^+$-dependent (ouabain-sensitive) phosphatase activity [37]; this observation was independent of the substrate used and of the actual rate of hydrolysis (Table VI). This contrasts not only with the ATPase activity but also with other processes in which $Mg^{2+}$ is required; for instance, in the promotion of vanadate binding, $Ca^{2+}$, although far less effective than $Mg^{2+}$, can still support binding to some degree [30].

Regarding the possible inhibition of the $Na^+$ pump by intracellular $Ca^{2+}$ in excitable cells during activity, from Fig. 2 it looks as if this would be rather minimal, if it happens at all.

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