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INTERACTION OF CALCIUM AND MAGNESIUM IN ACTIVATING AND INHIBITING THE NUCLEOSIDE TRIPHOSPHATASE OF SARCOPLASMIC RETICULUM VESICLES

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Summary

The dependence of ATP and ITP hydrolysis on the concentration of free Mg^{2+} and Ca^{2+} and of the metal · nucleotide complexes was investigated, using sarcoplasmic reticulum vesicles rendered leaky by treatment with ethyleneglycol-bis(aminoethyl)-tetraacetic acid at pH 9.5 or with diethylether. The Mg^{2+} · ATP complex is the true substrate of the reaction (K_m = 18.5 μM for Mg^{2+} · ATP concentrations higher than 6 μM), while the Ca^{2+} · ATP complex is a potent competitive inhibitor (K_i = 2 μM). At a fixed Mg^{2+} · ATP concentration, the optimal concentration of Ca^{2+} varies with the Mg^{2+} concentration. The inhibition in the presence of excess Ca^{2+} is caused by the Ca^{2+} · ATP complex. ITP hydrolysis, however, is not inhibited in the presence of excess Ca^{2+} (up to 1 mM), the Ca^{2+} · ITP complex not being inhibitory.

At low Ca^{2+} concentrations, in conditions which do not permit the formation of inhibitory levels of Ca^{2+} · ATP, hydrolysis of either nucleotide is activated by Ca^{2+}, showing a sigmoidal dependence on Ca^{2+} concentration. The apparent K_m for Ca^{2+} is not influenced by the concentration of Mg^{2+} · ATP or Mg^{2+} · ITP, and is practically the same in the presence of either nucleotide (0.23—0.28 μM). Mg^{2+} is a competitive inhibitor with respect to Ca^{2+}.

Introduction

It is well established that the Ca^{2+} transport shown by sarcoplasmic reticulum vesicles is driven by a (Ca^{2+} + Mg^{2+})-dependent nucleoside triphosphatase (EC 3.6.1.15) [1—6]. Kinetic properties of this system, and the pattern of its dependence on the divalent cations, have been studied by several authors, measuring either the hydrolysis of substrate, the formation of the phosphoprotein intermediate, or the incorporation of Ca^{2+} [7—14]. However, the formation of complexes between the divalent cations and the nucleotides has been

largely neglected. Although it has been proposed that the $Mg^{2+} \cdot$ ATP complex is the true substrate, this assertion has not been clearly demonstrated. Furthermore, there is much contradiction in the literature regarding the need for magnesium for the binding of ATP to sarcoplasmic reticulum vesicles and for formation of the phosphoprotein intermediate [10,13—19].

In this paper, the activation and inhibition of the sarcoplasmic reticulum vesicles (Ca^{2+} + Mg^{2+})-nucleoside triphosphatase by Mg^{2+}, Ca^{2+} and the nucleoside triphosphate complexes formed was investigated. The data show that the $Ca^{2+} \cdot$ ATP complex strongly inhibits the ATPase by competing with $Mg^{2+} \cdot$ ATP, the true substrate. Similar inhibition was not detected with the use of ITP.

Materials and Methods

Sarcoplasmic reticulum vesicles from rabbit skeletal muscle were prepared as described by de Meis and Hasselbach [20]. To avoid the inhibition of the ATPase by internal Ca^{2+} [8,21—22], as well as changes in external Ca^{2+} concentration, the vesicles were rendered incapable of retaining the transported Ca^{2+} by treatment with ethyleneglycol-bis(aminoethyl)-tetraacetic acid (EGTA) in alkaline pH [23]: EGTA and Tris · HCl buffer pH 9.5 were added to a suspension of sarcoplasmic reticulum vesicles (5—6 mg protein/ml) to final concentrations of 1 mM and 30 mM respectively. After 20 min at room temperature, the pH of the suspension was brought back to 7.0 with diluted HCl, and the preparation was used immediately. Some of the experiments were also performed with vesicles made leaky by pre-incubation with 7% (v/v) diethylether [9,24]. Essentially the same results were obtained using sarcoplasmic reticulum vesicles rendered leaky by these two procedures. Control tests showed that both kinds of treatment completely abolished the accumulation of Ca^{2+}, measured in the presence of 5 mM oxalate by the Millipore filtration technique [25], although the ATPase activity remained unimpaired.

Standard assay. The incubation media contained 30 mM Tris/maleate buffer, 20 mM KCl, 0.8—1 mM phosphoenolpyruvate, 0.06—0.12 mg/ml pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40), 0.2—0.5 mM EGTA, 0.05 mg/ml sarcoplasmic reticulum vesicle protein for the experiments with ATP and 0.15 mg/ml for those with ITP, and the concentrations of nucleoside triphosphate, $MgCl_2$, and $CaCl_2$ necessary for obtaining the desired concentrations of the complexes and free ionic species, calculated as described below. The ionic strength was adjusted to 0.15 with NH_4Cl. The pH of the media was carefully adjusted to 7.0. The KCl was included to activate pyruvate kinase. The use of higher concentrations of this salt was avoided since K^+ inhibits the ATPase in presence of low concentrations of either ATP or Ca^{2+} [26]. The incubations were carried out at 30° C. The reaction was started by addition of sarcoplasmic reticulum vesicles and stopped by addition of ice-cold trichloroacetic acid to a final concentration of 5% (w/v). The incubation time varied according to the composition of the medium from 2.5 to 40 min. Preliminary tests showed that the reaction was linear with time in the interval chosen. After centrifugation aliquots of the supernatants were assayed for orthophosphate by the method of Fiske and SubbaRow [27]. In all experiments, for each concen-

tration of Mg^{2+} · nucleoside triphosphate and/or Mg^{2+} tubes were run in parallel which contained 1 mM EGTA and no added Ca^{2+}, and the activity obtained (basic or Mg^{2+}-dependent) was subtracted from that obtained in the presence of Ca^{2+} to estimate the (Ca^{2+} + Mg^{2+})-dependent activity. The results of single experiments shown in the figures were confirmed in each case by at least three similar ones.

Nucleoside triphosphate regenerating system. The concentration of pyruvate kinase used to assure an adequate regeneration of the ATP and ITP hydrolysed was determined by the following procedure. In a first test the nucleoside triphosphatase activity was determined in the presence of increasing concentrations of pyruvate kinase and the fixed concentration of sarcoplasmic reticulum vesicle protein used routinely in the actual experiments. The activity always increased to a plateau level. The activity was subsequently measured in the presence of varying concentrations of sarcoplasmic reticulum vesicle protein in the range from half to twice the concentration mentioned above and in the presence of concentrations of pyruvate kinase that provided activities at the plateau level in the first test. The concentration of pyruvate kinase that permitted, in this second test, a straight line to be obtained in plots of the amount of P_i released as a function of the sarcoplasmic reticulum vesicle protein concentration, was chosen for use in the experiments. In this situation the specific activity of the nucleoside triphosphatase was independent of the sarcoplasmic reticulum vesicle protein concentration in the range mentioned, showing that maximal regeneration of the nucleoside triphosphate was achieved. These tests were performed for the different concentrations of ATP or ITP and salts used.

Calculation of the necessary concentrations of nucleoside triphosphate, $CaCl_2$ and $MgCl_2$. The equilibria considered for this calculation and the values chosen for the association constants are shown in Table I. The conservation equation for nucleoside triphosphate contained all its free and complexed forms shown in this table. The same applies to the other five conservation equations (for calcium, magnesium, potassium, EGTA and phosphoenolpyruvate required for the calculations. By simple algebraic manipulation of the equilibria in Table I, all forms of nucleoside triphosphate, EGTA and phosphoenolpyruvate were expressed in the conservation equations in terms of their fully deprotonated forms (e.g., $[HATP^{3-}] = K_1[H^+][ATP^{4-}]$; $[MgHATP^-] = K_2[Mg^{2+}]\ [HATP^{3-}] = K_1K_2[Mg^{2+}][H^+][ATP^{4-}]$; and so on). Extremely complex equations result if one seeks to obtain the concentrations of the free and complex forms from the total concentrations of all reactants. On the other hand, if the concentrations of free metallic species and NTP^{4-} are chosen in advance, the solution is simple. The total concentrations of EGTA and phosphoenolpyruvate can be defined. To define the concentrations of one or both of the complexes Mg^{2+} · nucleoside triphosphate and Ca^{2+} · nucleoside triphosphate, the following equations have to be considered first:

$$[MgNTP] = K_3[Mg^{2+}][NTP^{4-}]$$

$$[CaNTP] = K_4[Ca^{2+}][NTP^{4-}]$$

(where NTP is nucleoside triphosphate)
The term $[NTP^{4-}]$ is present in both equations. Of the five variables present in

TABLE I

VALUES FOR THE ASSOCIATION CONSTANTS USED IN THE COMPUTATIONS *

Abbreviations: PEP, phosphoenolpyruvate.

Reaction			Log K_a	References
H^+	+ $HATP^{3-}$	⇌ H_2ATP^{2-}	4.02	28,29
H^+	+ ATP^{4-}	⇌ $HATP^{3-}$	7.02 (K_1)	30,31
K^+	+ ATP^{4-}	⇌ $KATP^{3-}$	1.11	32,31
Mg^{2+}	+ $HATP^{3-}$	⇌ $MgHATP^-$	2.65 (K_2)	28,33
Mg^{2+}	+ ATP^{4-}	⇌ $MgATP^{2-}$	4.65 (K_3)	28,33,34,35,36,37
Ca^{2+}	+ $HATP^{3-}$	⇌ $CaHATP^-$	2.13	28
Ca^{2+}	+ ATP^{4-}	⇌ $CaATP^{2-}$	4.32 (K_4)	28,35,36
H^+	+ PEP^{3-}	⇌ $HPEP^{2-}$	6.35	38
K^+	+ PEP^{3-}	⇌ $KPEP^{2-}$	1.08	38
Mg^{2+}	+ PEP^{3-}	⇌ $MgPEP^-$	2.26	38
Ca^{2+}	+ PEP^{3-}	⇌ $CaPEP^-$	2.08	**
H^+	+ $HEGTA^{3-}$	⇌ H_2EGTA^{2-}	8.85	40
H^+	+ $EGTA^{4-}$	⇌ $HEGTA^{3-}$	9.43	40
Mg^{2+}	+ $HEGTA^{3-}$	⇌ $MgHEGTA^-$	3.36	41
Mg^{2+}	+ $EGTA^{4-}$	⇌ $MgEGTA^{2-}$	5.20	40
Ca^{2+}	+ $HEGTA^{3-}$	⇌ $CaHEGTA^-$	5.32	41
Ca^{2+}	+ $EGTA^{4-}$	⇌ $CaEGTA^{2-}$	11.00	40

* The same values were considered for ATP and ITP [36,37].

** Approximate value assuming a ratio of 1.5 for the constants for Mg^{2+} and Ca^{2+}, as reported for the Mg^{2+} and Ca^{2+} complexes with other phosphate compounds [39].

these two equations, a value for at most three of them can be chosen independently, the value of the other two being then automatically determined and easily obtained. The conservation equation for EGTA can be solved for [$EGTA^{4-}$] using the assigned values for the free metallic species, allowing subsequently the calculation of all other EGTA forms. Similar reasoning applies to phosphoenolpyruvate. A program was prepared for a Hewlett-Packard 9810A calculator to perform the calculations. Therefore one enters in the calculator the concentrations of Mg^{2+}, Ca^{2+}, NTP^{4-} (defined at will or obtained from the equations above after defining values for one or both the complexes), K^+, H^+, EGTA and phosphoenolpyruvate, obtaining the concentrations of all species involved and the total concentrations of nucleoside triphosphate and metal salts that must be used in the reaction mixture. A few examples of the calculations are presented in Table II. Free ATP is used in the results to refer to all forms of ATP not complexed with metals; at pH 7.0 it is a mixture, practically equimolecular, of ATP^{4-} and $HATP^{3-}$. As the concentrations of the potassium complexes are a small fraction of the total potassium, the KCl was held constant (20 mM) to a simplify the preparation of the reaction mixtures. Although all the experiments described in this paper were done at pH 7.0, the equilibria considered and the program prepared allow the calculations for any pH above 5.0.

As the program does not allow a direct calculation of the concentrations of the complexes and free species from the total concentrations of ATP and salts, in the experiment shown in Fig. 1 the former values were found by trial and error, feeding the calculator values for the concentrations of ATP^{4-} and

TABLE II

FREE, COMPLEXED AND TOTAL CONCENTRATIONS OF ATP, METALS, EGTA AND PHOSPHOENOLPYRUVATE FOR SELECTED POINTS FROM FIG. 3

Calculation as described in the text. Each vertical column represents a different point; compare Ca^{2+} in table with abscissa of Fig. 3A and Mg^{2+} with legend of figure for identification of points. All concentration values in the table are in μM. Total Mg and total Ca represent the concentration of their chloride salts used in the reaction mixture. Total concentrations of EGTA and PEP were 0.5 mM and 0.8 mM respectively. At pH 7.0, free EGTA is a mixture of $EGTA^{4-}$, $HEGTA^{3-}$ and H_2EGTA^{2-} in the ratio 1 : 71 : 19 170. Free PEP is a mixture of PEP^{3-} and $HPEP^{2-}$ in the ratio 4.46 : 1. MgEGTA refers to the total of $MgEGTA^{2-}$ plus $MgEGTA^{-}$, in the ratio 1 : 3.83.

$MgATP^{2-}$	100	100	100	100	100	100
$MgHATP^{-}$	1.05	1.05	1.05	1.05	1.05	1.05
$CaATP^{2-}$	23.33	0.467	4.67	0.009	9.33	2.33
$CaHATP^{-}$	0.16	0.003	0.032	<0.001	0.063	0.016
$KATP^{3-}$	28.88	5.77	5.77	1.15	1.15	0.29
ATP^{4-}	111.1	22.22	22.22	4.44	4.44	1.111
$HATP^{3-}$	116.7	23.33	23.33	4.67	4.67	1.117
Total ATP	381.3	152.9	157.1	111.3	120.7	106
Mg^{2+}	20	100	100	500	500	2000
MgEGTA	0.008	0.327	0.038	6.56	0.02	0.078
$MgPEP^{-}$	1.96	9.27	9.71	46.3	46	156.9
Total Mg	123	211.1	210.8	653.9	647	2258
Ca^{2+}	10	1	10	0.1	100	100
$CaEGTA^{2-}$	490.2	418.2	490.2	167.6	498.7	498.7
$CaHEGTA^{-}$	0.28	0.24	0.28	0.095	0.28	0.28
$CaPEP^{-}$	0.65	0.065	0.65	0.006	6.13	5.23
Total Ca	524.6	419.9	505.8	167.8	614.5	606.5
Free EGTA	9.53	81.3	9.53	325.8	0.97	0.97
$KPEP^{2-}$	130.7	129.5	129.4	123.6	122.6	104.6
Free PEP	625.3	660.7	660.2	630.1	625.3	533.3

free cations until the total concentrations of ATP and salts used for the experiment were obtained.

Standardization of solutions of nucleoside triphosphate and metal salts. Solutions of ATP and ITP were standardized spectrophotometrically [42—43]. Solutions of the chlorides of Ca^{2+} and Mg^{2+} were standardized by titration with the disodium salt of EDTA [40], using calcein as the indicator for Ca^{2+} and Indicator Buffer Tablets (E. Merck, Darmstadt) for Mg^{2+}.

Calculation of regression lines and Hill coefficients. All straight lines in the double-reciprocal plots are weighted regression lines, calculated as described by Johansen and Lumry [44], assuming that the standard deviation of v is proportional to v. Due to the difficulty in obtaining precise values for V, the Hill coefficients (n) for the experiments of the type shown in Figs 6, 7, 8 and 10 were obtained by an improved version of a procedure suggested by Ting-Beall et al. [45]. The program used for the calculation of the weighted regression lines was adapted in such a way that the Ca^{2+} concentrations were first elevated to the nth power. Then the value of n was varied until the value which made the variance of $1/v$ closest to zero was obtained. This value provides the best fit of a weighted regression line to the experimental points in double-reciprocal

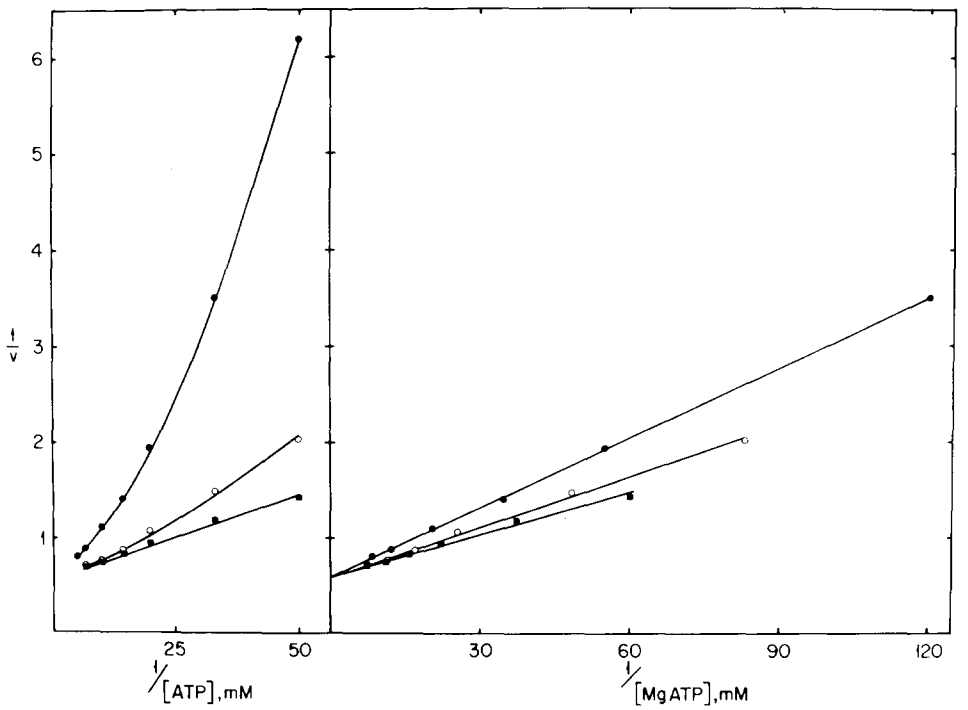

Fig. 1. Double-reciprocal plot of the activity as a function of the concentration of total ATP (left) or of the concentration of Mg^{2+} · ATP complex present in the reaction mixture (right). The relationships of the concentrations of $MgCl_2$ and ATP were: (●) equimolecular concentrations; (○) $MgCl_2$ concentration 5-fold greater than the ATP concentration; (■) $MgCl_2$ in constant excess of 0.5 mM over the ATP concentration. Free Ca^{2+} concentration was 1 μM.

plots in which $1/[Ca^{2+}]^n$ is placed on the abscissa, and was taken to represent the best value for n. As a counter-check, values of V obtained from such plots were used for the calculation of n from standard Hill plots. The values of n obtained were in good agreement with the ones obtained by the procedure described.

Results

Mg^{2+} · ATP complex as the true substrate

The aim of the first set of experiments was to ascertain whether magnesium activates the enzyme in its ionic form or by forming the Mg^{2+} · ATP complex. If this complex is the true substrate, one would expect non-linear, double-reciprocal plots of the activity as a function of the added ATP concentration, when one varies the concentrations of ATP and $MgCl_2$ in equimolecular fashion, because the proportion of the nucleotide present as the complex decreases as the total concentrations of the components decrease. This behavior is observed in Fig. 1A (upper curve). However, a straight line should be obtained if the plot is made as a function of the concentration of the Mg^{2+} · ATP complex, as in Fig. 1B. In the presence of an excess of $MgCl_2$, the data should approach a straight line even when plotted as a function of total ATP (Fig. 1A),

since most of the nucleotide should now be in the complex form. In these experiments the Ca^{2+} concentration was kept constant at 1 μM. The small differences in slopes observed in Fig. 1B are probably related to different concentrations of the Ca^{2+} · ATP complex present, as will be shown below.

A break in double-reciprocal plots was observed when the Mg^{2+} · ATP concentration was varied over a broad range (from 0.2 μM to 1 mM) (Fig. 2). A first straight line was observed in the range from 0.2 to 3 μM, giving an apparent K_m of 1.1 μM ± 0.15 (S.E.) (6 different sarcoplasmic reticulum vesicle preparations). The range of Mg^{2+} · ATP concentrations of the experiments shown in Figs 1 and 5 fall within the second straight line, which showed an apparent K_m of 18.5 μM ± 2.0 (S.E.) (11 different sarcoplasmic reticulum vesicle preparations). The appearance of two K_m values for Mg^{2+} · ATP in this range is consistent with the finding by Yamamoto and Tonomura [10] of a break in double-reciprocal plots of the activity vs ATP concentration in the presence of a large excess of magnesium.

Relationship of the optimal concentrations of Mg^{2+} and Ca^{2+} at a fixed concentration of Mg^{2+} · ATP

The optimal concentrations of Ca^{2+} and Mg^{2+} for activating the enzyme were interdependent. As shown in Fig. 3A, at the lowest concentration of Ca^{2+} (0.1 μM) increasing concentrations of Mg^{2+} were inhibitory. As the concentra-

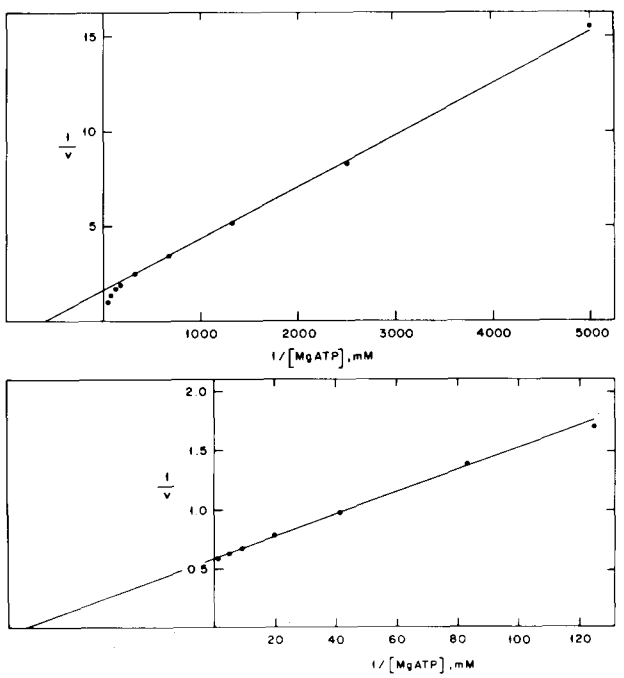

Fig. 2. Double-reciprocal plot of the activity as a function of the concentration of Mg^{2+} · ATP complex. The concentration of Mg^{2+} was 0.5 mM and of Ca^{2+} 1 μM. The four points for the higher Mg^{2+} · ATP concentrations on the upper graph were not considered for the calculation of the weighted regression line. The three highest concentrations shown in the upper graph are replotted as the lower concentrations in the lower graph.

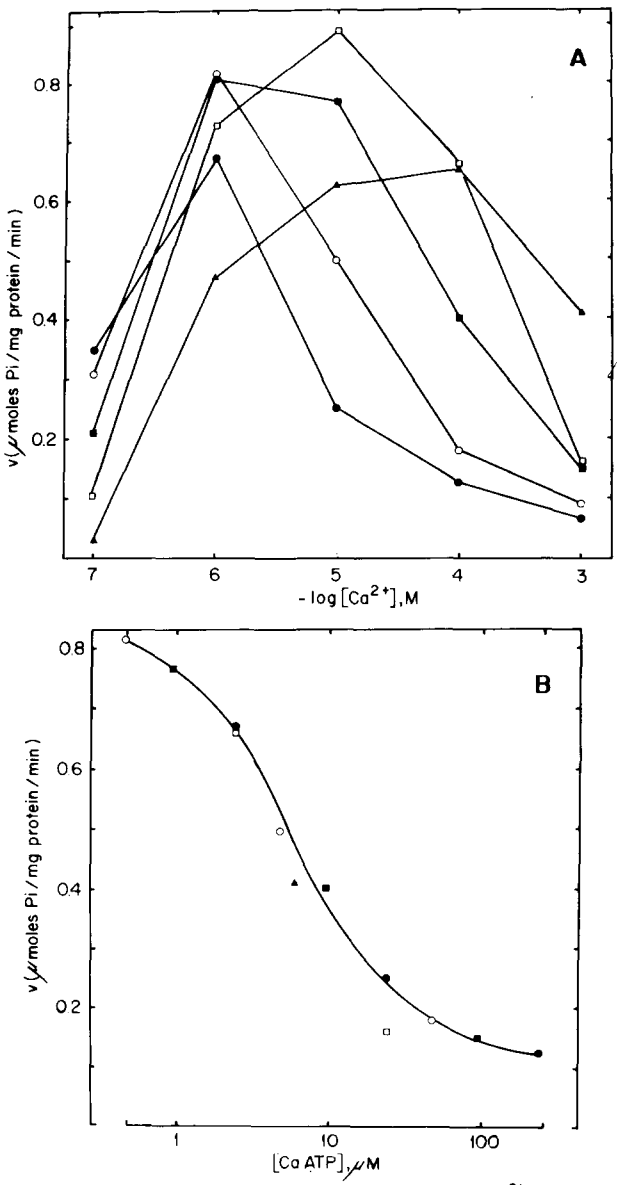

Fig. 3. (A), Dependence of the activity on the free Ca^{2+} concentration, at different levels of free Mg^{2+}: (●) 0.02 mM; (○) 0.1 mM; (■) 0.5 mM; (□) 2 mM; (▲) 8 mM. The Mg^{2+} · ATP concentration was 0.1 mM. In each curve the concentration of Ca^{2+} · ATP increases as the Ca^{2+} increases but the concentration of free ATP is constant at (●) 228 μM; (○) 45.6 μM; (■) 9.12 μM; (□) 2.28 μM; (▲) 0.57 μM; (B) Points from (A) where inhibition by excess of Ca^{2+} is manifest plotted as a function of the concentration of the Ca^{2+} · ATP complex present in the reaction mixture. The symbols indicate from which curves of (A) the points were taken.

tion of Ca^{2+} was increased, this inhibition was reversed. The optimal Ca^{2+} concentration depended upon the concentration of Mg^{2+}, increasing with that of Mg^{2+}. Concentrations of Ca^{2+} greater than optimal appeared to be inhibitory.

Responsibility of the Ca^{2+} · ATP complex for inhibition by "excess" Ca^{2+}

Analysis of the results of experiments of the type shown in Fig. 3A indicated that points on different curves which showed approximately the same level of activity at concentrations of Ca^{2+} higher than optimal, had in the

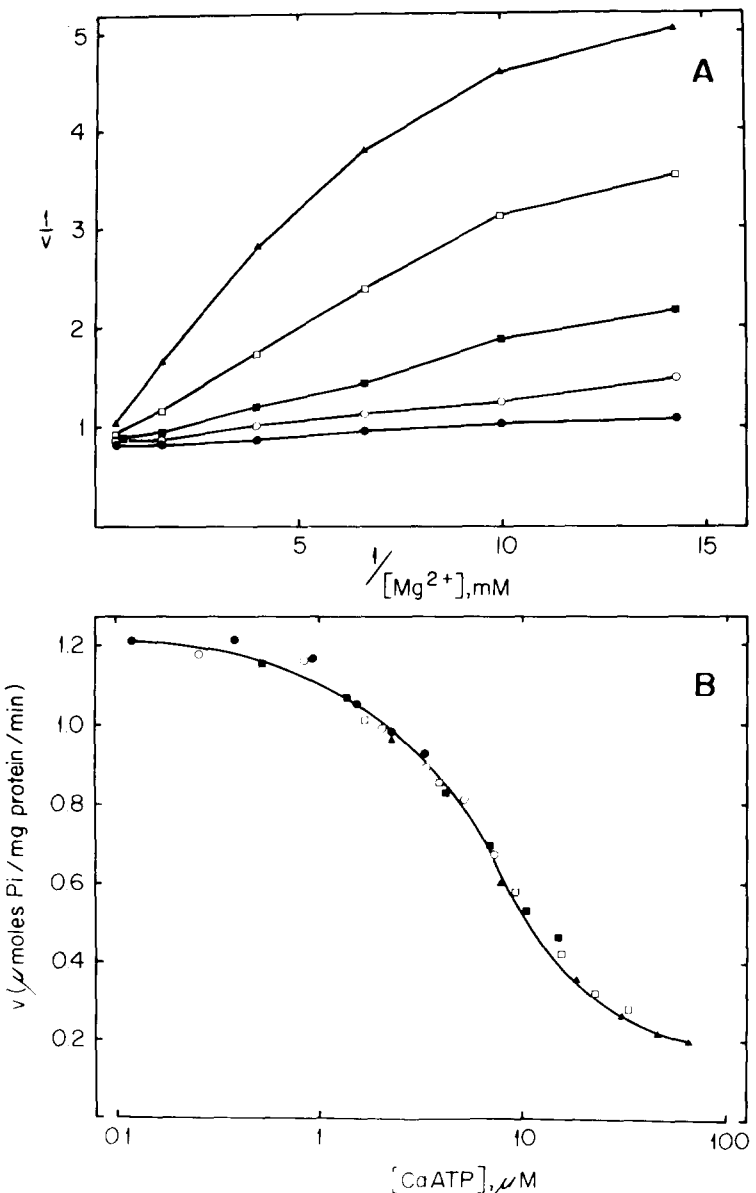

Fig. 4. (A) Double-reciprocal plot of the activity as a function of the concentration of Mg^{2+}, at different inhibitory Ca^{2+} concentrations: (●) 10μM; (○) 22 μM; (■) 45 μM; (□) 100 μM; (▲) 200 μM; The Mg^{2+} · ATP concentration was 50 μM. Free ATP is constant at each concentration of Mg^{2+}, varying from 32.6 μM (at 0.07 mM Mg^{2+}) to 1.14 μM (at 2 mM Mg^{2+}). (B) all points of (A) plotted against the concentration of the Ca^{2+} · ATP complex present in the reaction mixture. The symbols indicate to which curves of (A) the points belonged.

reaction mixture similar concentrations of the Ca^{2+} · ATP complex. A single curve is obtained (Fig. 3B) plotting the points in Fig. 3A where the inhibition by "excess" Ca^{2+} was manifested, as a function of the concentration of the Ca^{2+} · ATP complex. This accurate fit suggests that the observed inhibition is caused by this complex.

The next experiment was designed to test the possibility that magnesium, besides forming a complex with ATP, could also activate the reaction by binding to the enzyme. In this hypothesis excess of Ca^{2+} could inhibit the reaction by displacing the activating Mg^{2+} [11—13,16]. Fig. 4A shows a double-reciprocal plot of the activity as a function of Mg^{2+} concentration, at several levels of excess Ca^{2+}. The Mg^{2+} · ATP concentration was kept constant at 50 μM. The curves obtained were not linear, and the general pattern is rather complicated. However, if all the points in this experiments are plotted as a function of the concentration of the Ca^{2+} · ATP complex (Fig. 4B), one observes again behavior that is consistent with the inhibition being caused by this complex.

As shown in Fig. 5, the Ca^{2+} · ATP complex inhibits the reaction by competing with the substrate Mg^{2+} · ATP. This type of experiment can be done by fixing the concentration of one of the divalent cations and varying the concentration of the other, to obtain the desired levels of the metal · ATP

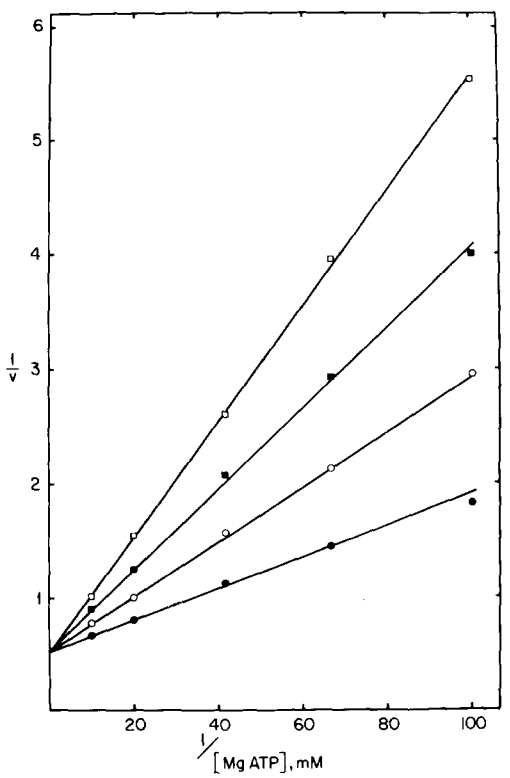

Fig. 5. Double-reciprocal plot of the activity as a function of the Mg^{2+} · ATP concentration, at several levels of Ca^{2+} · ATP complex. (●) 0.3 μM; (○) 1.5 μM; (■) 3.5 μM; (□) 8 μM. The Ca^{2+} concentration was 15 μM. The concentration of Mg^{2+} is different at each point (see text).

complexes. In the experiment shown, the Ca^{2+} concentration was held constant at 15 μM, Mg^{2+} varying from 8.8 μM to 2.33 mM. For each of the four concentrations of Ca^{2+} · ATP, the concentration of free ATP was constant, being 1.95 μM at 0.3 μM Ca^{2+} · ATP and 52.1 μM at 8 μM Ca^{2+} · ATP. Conversely, when the Mg^{2+} was fixed at 0.2 mM and the Ca^{2+} concentration varied in a range of 1.3 to 343 μM, similar results were obtained. In this case the free ATP was constant for each level of Mg^{2+} · ATP, varying from 2.28 μM (at 10 μM Mg^{2+} · ATP) to 22.8 μM (at 100 μM Mg^{2+} · ATP). These observations suggest that free ATP does not play a role in the reaction, at least in this concentration range. The value obtained for the apparent K_i for Ca^{2+} · ATP was 2.04 μM ± 0.16 (S.E.) (13 experiments, in presence of different levels of Ca^{2+} · ATP, using 5 sarcoplasmic reticulum vesicle preparations). The values were calculated taking the apparent K_m for Mg^{2+} · ATP obtained in the presence of the lowest Ca^{2+} · ATP concentration used in each experiment (usually 0.1 or 0.3 μM).

The activation by Ca^{2+}

Turning now to the range of Ca^{2+} concentrations which activate ATP hydrolysis, the dependence of the activity on the Ca^{2+} concentration was studied, at fixed concentrations of Mg^{2+} · ATP and Mg^{2+} and with Ca^{2+} · ATP concentrations too low to be inhibitory. The curves obtained showed sigmoidicity (Fig. 6). The value of the Hill coefficient (n) was calculated as described in Methods. In 48 curves obtained in the presence of different concentrations of Mg^{2+} · ATP (from 1 μM to 2 mM) and Mg^{2+} (from 0.1—3 mM), using

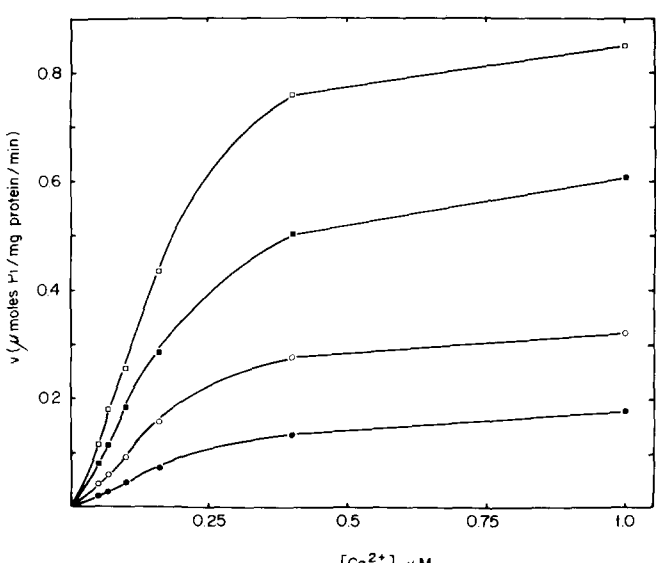

Fig. 6. ATP hydrolysis as a function of the Ca^{2+} concentration, at different Mg^{2+} · ATP concentrations: (●) 1.5 μM; (○) 6 μM; (■) 24 μM; (□) 100 μM. The Mg^{2+} concentration was 0.5 mM. Maximal Ca^{2+} · ATP concentrations (at 1 μM Ca^{2+}) were: (●) 1.4 nM; (○) 5.6 nM; (■) 22.4 nM; (□) 93.3 nM. Similar curves were obtained with Mg^{2+} · ATP concentrations up to 2 mM.

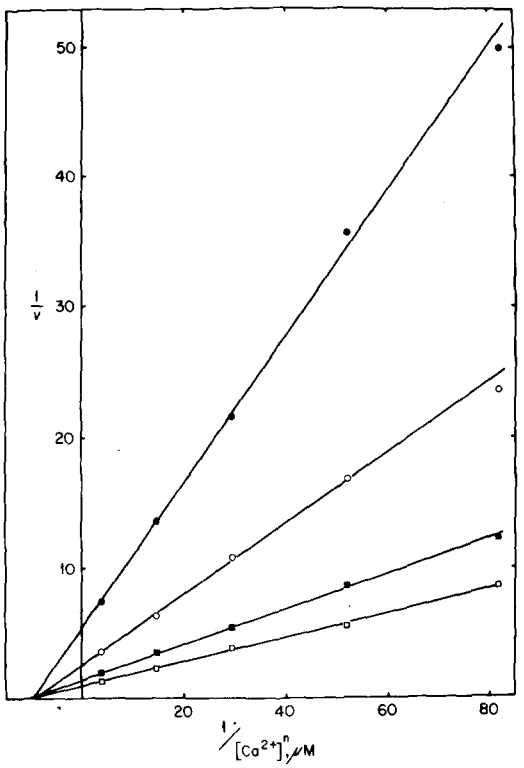

Fig. 7. Double-reciprocal plot of the data shown in Fig. 6, as a function of the Ca^{2+} concentration elevated to the nth power. $n = 1.5$. Value of n obtained as described in Methods. The symbols are the same as in Fig. 6.

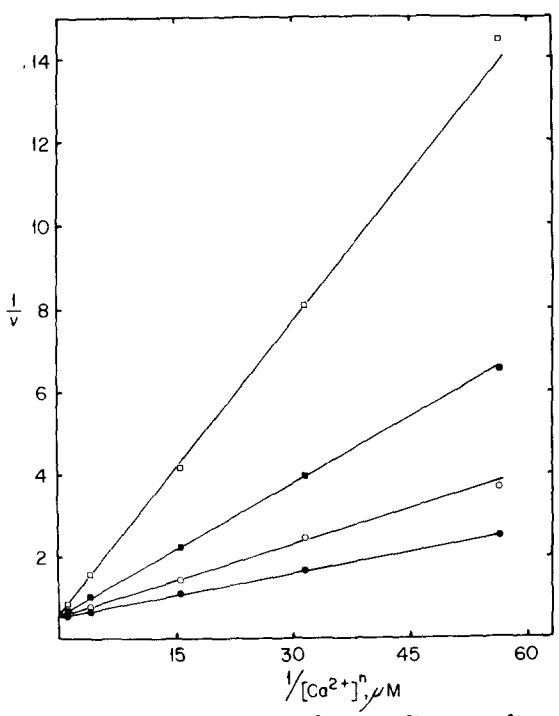

Fig. 8. Competition between Mg^{2+} and Ca^{2+}. The Mg^{2+} concentrations were: (●) 0.1 mM; (○) 0.5 mM; (■) 1.5 mM; (□) 3 mM. The Mg^{2+}· ATP concentration was constant at 0.1 mM. $n = 1.47$. The highest Ca^{2+} · ATP concentration was 0.47 μM (at 0.1 mM Mg^{2+}, 1 μM Ca^{2+}).

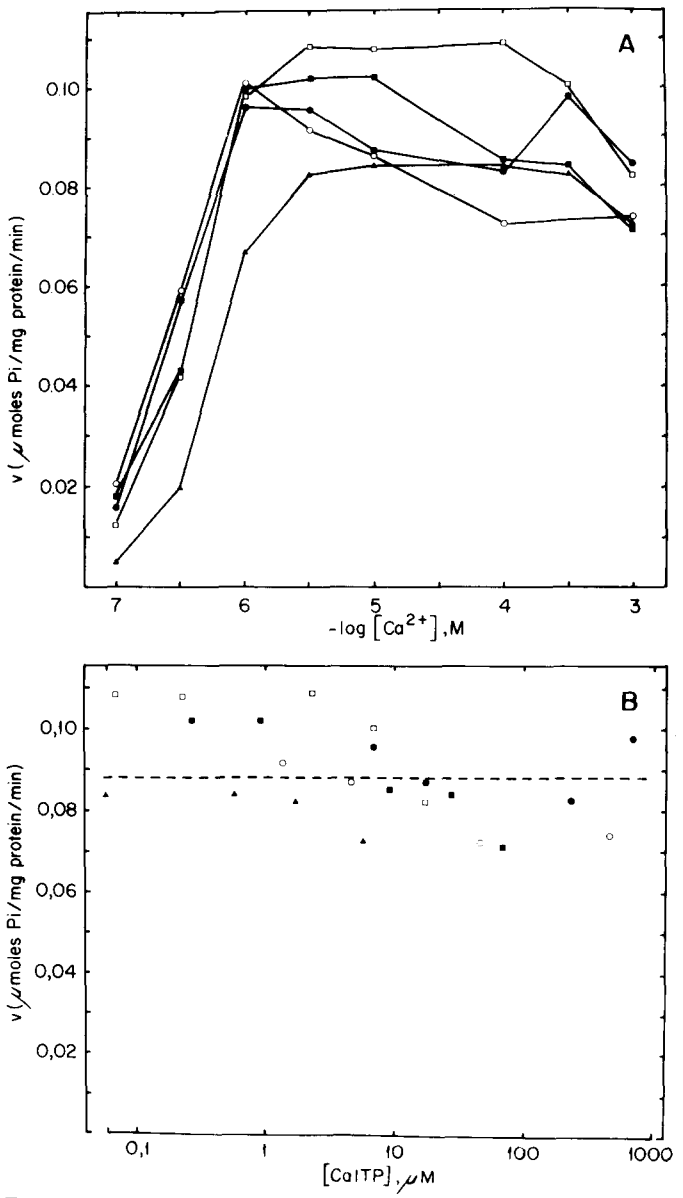

Fig. 9 (A) Dependence of the activity on the Ca^{2+} concentration, at different Mg^{2+} concentrations, using Mg^{2+} · ITP as substrate in place of Mg^{2+} · ATP. All other experimental conditions and symbols were the same as in Fig. 3. (B) points from (A) in which the Ca^{2+} concentration is 3 μM or higher, plotted as a function of the concentration of the Ca^{2+} · ITP complex present in the reaction mixture. The dashed line was drawn to indicate the average of the activity values.

6 sarcoplasmic reticulum vesicle preparations, the average value of n was 1.59 ± 0.03 (S.E.).

Fig. 7 shows that the apparent K_m for Ca^{2+} does not vary with the Mg^{2+} · ATP concentration. In the presence of 0.5 mM Mg^{2+}, and a range of Mg^{2+} · ATP concentration from 1 μM to 2 mM, its value was 0.230 μM ± 0.008 (S.E.), in 31

experiments performed with 4 different sarcoplasmic reticulum vesicle preparations.

Competition between Mg^{2+} and Ca^{2+}

Fig. 8 shows that the inhibition of the activity by increasing Mg^{2+} concentrations which is observed at suboptimal concentrations of Ca^{2+} (see Fig. 3) is competitive.

Dependence of ITP hydrolysis on the Mg^{2+} and Ca^{2+} concentrations

The dependence of the activity on the concentrations of Mg^{2+} and Ca^{2+} when Mg^{2+} · ITP was used as substrate is shown in Fig. 9. The concentrations of cations are the same as those in Fig. 3. The most striking feature of this figure is the absence of inhibition in the presence of excess Ca^{2+}, at least in the concentration range studied. Athough the Ca^{2+} · ITP complex is also formed, since the association constants for Ca^{2+} and Mg^{2+} of both ITP and ATP are practically the same [36,37] the activity was now independent of the level of Ca^{2+} · ITP present, from 0.1 μM to 1 mM (Fig. 9B).

The curve of the activity as a function of Ca^{2+} concentration was also sigmoidal when the substrate was Mg^{2+} · ITP. Fig. 10 shows that the apparent K_m for Ca^{2+} was not changed either by the Mg^{2+} · ITP concentration. Further-

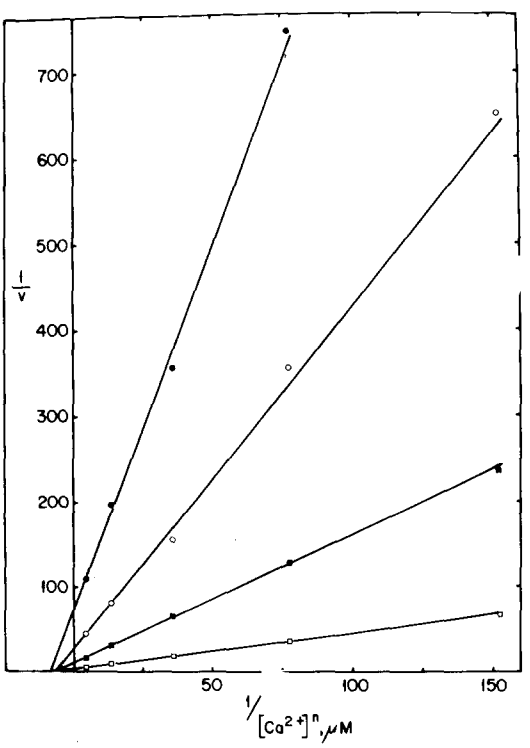

Fig. 10. Double-reciprocal plot of the activity as a function of the concentration of Ca^{2+} elevated to the nth power, at different Mg^{2+} · ITP concentrations: (●) 6 μM; (○) 24 μM; (■) 0.1 mM; (□) 0.75 mM. The Mg^{2+} concentration was 1 mM. n = 1.89.

more, the value found, 0.282 μM ± 0.013 (S.E.) (13 determinations) was quite close to the one observed using Mg^{2+} · ATP as substrate.

It will be noted that the values found here for the ITPase activity are much lower than those obtained for ATP hydrolysis. At the concentrations of nucleoside triphophates used here, this is consistent with the data reported that ITP is a substrate of much lower affinity than ATP [6] and that using high concentrations of these nucleotides the initial rate of Ca^{2+} uptake and P_i liberation is 2—3 times greater in the presence of ATP [6,46].

Discussion

The observations presented in this paper regarding the interaction of calcium and magnesium for activating the (Ca^{2+} + Mg^{2+})-dependent ATPase of sarcoplasmic reticulum vesicles can be summarized in the following way: (1) magnesium activates the reaction by forming the Mg^{2+} · ATP complex, which is the true substrate. Free Ca^{2+} activates the reaction. At low Ca^{2+} concentrations, excess of Mg^{2+} inhibits the reaction by competing with Ca^{2+}. (2) High concentrations of calcium bring about inhibition through the formation of the Ca^{2+} · ATP complex, which competes with the substrate Mg^{2+} · ATP. At a given Ca^{2+} concentration, increasing Mg^{2+} reverses the inhibition by decreasing the concentration of the inhibitory complex. This effect is not observed when ITP is used as substrate. It should be pointed out that these conclusions are valid only for the range of divalent cation concentrations studied (up to 1 mM Ca^{2+} and 8 mM Mg^{2+}), and an incubation temperature of 30° C. It is possible that other types of effects would be revealed at higher concentrations of salts or different incubation temperatures [47—48].

Inhibition by excess Mg^{2+}

The ratios of the concentrations of Mg^{2+} and Ca^{2+} for which this inhibition is observed (Figs 3A and 8) indicate that the affinity for Mg^{2+} is at least three orders of magnitude lower than for Ca^{2+}. Weber et al [8] failed to observe any inhibition of Ca^{2+} uptake by Mg^{2+} concentration up to 10 mM. Some inhibition of the ATPase by excess of Mg^{2+} can be observed, however, in data presented by other authors [5,9]. Yamada and Tonomura [12] also observed competition between Mg^{2+} and Ca^{2+} determining the rate of phosphoprotein formation. The observation that Mg^{2+} increases the dissociation constant for the passive binding of Ca^{2+} to sarcoplasmic reticulum versicles [19,49] may also be pertinent to the findings reported here.

Inhibition in the presence of excess Ca^{2+}

Inhibition of ATP hydrolysis by high Ca^{2+} concentrations has been shown by several authors, using intact [7,12,16,50] as well as solubilized or ether-treated vesicles [9,22,48]. However, no attempt has been made to evaluate the role played in this inhibition by the Ca^{2+} · ATP complex, whose concentration increases with the increase in Ca^{2+} concentration. The data in Figs 3B, 4B and 5 show that the membrane displays an affinity for Ca^{2+} · ATP at least 8 times higher than for Mg^{2+} · ATP, Ca^{2+} · ATP not being hydrolyzed. Competition between Ca^{2+} · ATP and Mg^{2+} · ATP has also been indicated to be the mecha-

nism of the inhibition of the (Na^+ + K^+)ATPase brought about by calcium [51,52].

Studying the phosphoprotein intermediate formed in the process of ATP hydrolysis, several authors have postulated a binding site in the membrane for Mg^{2+} [11—13,16]. According to this view, Mg^{2+} would activate ATP hydrolysis by accelerating the hydrolysis of the phosphoprotein and excess of Ca^{2+} would inhibit by competing with Mg^{2+} for this site. Our results show that in presence of ITP, no inhibition by Ca^{2+} up to 1 mM is observed. If there was a competition between Ca^{2+} and Mg^{2+} in the dephosphorylation step, in the concentration range of metals studied, one should expect similar inhibition regardless of which nucleoside triphosphate was used as substrate.

A puzzling observation in this paper is the non-inhibitory effect of the Ca^{2+} · ITP complex. This may be related to the difference of affinity reported for ATP and ITP as substrates [6].

The Ca^{2+} affinity

The value observed for the apparent K_m for Ca^{2+}, in the range of 0.2—0.3 μM, is very close to values reported by other authors for ATP hydrolysis, accumulation of Ca^{2+} or phosphoprotein formation [8,9,11,14,21]. Similar values have also been reported for the dissociation constant of passive Ca^{2+} binding [48,49,53]. The value of 1.6 found for the Hill coefficient is consistent with the ratio of two Ca^{2+} transported for each ATP molecule hydrolyzed [5,8,11,54] and indicates the possible existence of cooperativity for Ca^{2+} binding.

Possible effect of temperature

Recently it has been reported that sarcoplasmic reticulum vesicles behave differently at 0°C than at 37°C. At 37°C the level of membrane phosphorylation by ATP is decreased in the presence of excess Ca^{2+}. This inhibition is abolished when the temperature of the assay is decreased to 0°C. With the use of ITP, no inhibition by excess Ca^{2+} was observed, regardless of the assay temperature [6,55]. The findings at 37°C are in agreement with the data presented in this paper regarding the inhibition by Ca^{2+} · ATP and lack of inhibition by Ca^{2+} · ITP. It might be possible that an effect of temperature is related to an apparent discrepancy between findings reported in this paper and data of de Meis and de Mello [6]. Studying the membrane phosphorylation by ITP and ATP at 0°C, they found that the Ca^{2+} affinity varies with the concentration of ITP, being similar to that in the presence of ATP only in a high concentration of ITP (1 mM). The data in Figs 7 and 10 show that at 30°C the apparent K_m for Ca^{2+} does not vary with the nucleoside triphosphate or its concentration.

Inhibition by internal Ca^{2+}

It should be pointed out that the inhibition of ATP hydrolysis by excess Ca^{2+} reported in this paper and shown to be due to competition by the Ca^{2+} · ATP complex is probably not related to the inhibition by high Ca^{2+} at the internal surface of closed vesicles [8,21—22]. This latter inhibition is probably associated with the low affinity Ca^{2+} binding sites observed by Ikemoto [48] in

solubilized vesicles at 0°C. Indeed, there is evidence for additive inhibitory effects of high Ca^{2+} inside and outside closed vesicles [22]. Although the inhibition by high internal Ca^{2+} in closed vesicles is seen with ITP as well as with ATP [22], no inhibition of ITP hydrolysis was seen in this paper using Ca^{2+} below 1 mM. It is probable therefore that in leaky vesicles, at 30°C, the Ca^{2+} concentrations needed to affect the sites postulated to be responsible for the inhibition by internal Ca^{2+} are higher than 1 mM.

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