Biochimica et Biophysica Acta, 557 (1979) 230—242

BBA 78517

Mg^{2+} AND ATP EFFECTS ON K^+ ACTIVATION OF THE Ca^{2+}-TRANSPORT ATPase OF CARDIAC SARCOPLASMIC RETICULUM

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(Received February 21st, 1979)

Key words: Cardiac sarcoplasmic reticulum; Ca^{2+}-transport ATPase; K^+ activation

Summary

ATP and the divalent cations Mg^{2+} and Ca^{2+} regulated K^+ stimulation of the Ca^{2+}-transport ATPase of cardiac sarcoplasmic reticulum vesicles. Millimolar concentrations of total ATP increased the K^+-stimulated ATPase activity of the Ca^{2+} pump by two mechanisms. First, ATP chelated free Mg^{2+} and, at low ionized Mg^{2+} concentrations, K^+ was shown to be a potent activator of ATP hydrolysis. In the absence of K^+ ionized Mg^{2+} activated the enzyme half-maximally at approximately 1 mM, whereas in the presence of K^+ the concentration of ionized Mg^{2+} required for half-maximal activation was reduced at least 20-fold. Second MgATP apparently interacted directly with the enzyme at a low affinity nucleotide site to facilitate K^+-stimulation. With a saturating concentration of ionized Mg^{2+}, stimulation by K^+ was 2-fold, but only when the MgATP concentration was greater than 2 mM. Hill plots showed that K^+ increased the concentration of MgATP required for half-maximal enzymic activation approx. 3-fold.

Activation of K^+-stimulated ATPase activity by Ca^{2+} was maximal at an ionized Ca^{2+} concentration of approx. 1 μM. At very high concentrations of either Ca^{2+} or Mg^{2+}, basal Ca^{2+}-dependent ATPase activity persisted, but the enzymic response to K^+ was completely inhibited. The results provide further evidence that the Ca^{2+}-transport ATPase of cardiac sarcoplasmic reticulum has distinct sites for monovalent cations, which in turn interact allosterically with other regulatory sites on the enzyme.

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

Introduction

Sarcoplasmic reticulum fractions isolated from myocardium actively transport Ca^{2+}. Recent work suggests that both the rate and extent of Ca^{2+}-transport by the Ca^{2+}-pump of cardiac sarcoplasmic reticulum vesicles is regulated by monovalent cations [1—4]. Stimulation of activity by monovalent cations can be demonstrated by measuring Ca^{2+}-dependent ATP hydrolysis, Ca^{2+} uptake, or the kinetics of decomposition of the radioactively labelled phosphoenzyme intermediate [1—4]. Skeletal muscle sarcoplasmic reticulum also contains a Ca^{2+}-transport ATPase which is stimulated by monovalent cations [5,6]. For membrane preparations from either tissue, K^+ is the most potent of a series of monovalent cations which have been tested [1,2,5,6]. K^+ apparently increases the turnover of the Ca^{2+}-pump by accelerating the rate of hydrolysis of the acylphosphoprotein enzyme intermediate, which is the rate-limiting step in the overall reaction mechanism [2—4,6,7].

In a recent study we observed that the magnitude of the enzymic activity simulated by K^+ in cardiac preparations depended on the ATP concentration [2], optimal stimulation requiring millimolar concentrations of ATP. This effect has not been found for skeletal muscle preparations [5,7]. However, in the earlier study it was also apparent that the ionized Mg^{2+} concentration could alter stimulation of activity by K^+, and that the ionized Mg^{2+} concentration in turn was dependent on the total concentration of ATP used [2]. In the present report we have examined in more detail Mg^{2+} and ATP effects on K^+-stimulation of the Ca^{2+}-dependent ATPase activity of cardiac sarcoplasmic reticulum. The results suggest that distinct sites on the enzyme are present for each of the activators.

Experimental procedures

Preparation of cardiac sarcoplasmic reticulum vesicles

Cardiac microsomes were isolated from canine ventricle [8]. 25—30 g myocardium were homogenized three times for 30 s with a Polytron PT-20 (Brinkman Instruments) at half-maximal speed in 100 ml 10 mM $NaHCO_3$ [9]. Large particles were sedimented by two sequential centrifugations at 14 000 × g_{max} for 20 min. Microsomes were pelleted from the second supernatant by centrifugation at 44 000 × g_{max} for 30 min. Contractile proteins were subsequently extracted in 0.6 M KCl and 30 mM histidine (pH 7.0) and the final membrane suspension was stored frozen at −20°C in 0.25 M sucrose and 30 mM histidine (pH 7.0). Virtually all Ca^{2+}-dependent ATPase activity in the preparation is contributed by membranes originating from sarcoplasmic reticulum [10,11].

Assay of ATPase activity

ATP hydrolysis was monitored by following the production of P_i from ATP colorimetrically [8]. All assays were conducted in 50 mM histidine at pH 7.0 at a temperature of 37°C with 15—20 μg of membrane protein present in 1 ml of reaction medium. Ca^{2+}-dependent ATPase activity is defined as the difference between total ATPase activity (measured in the presence of Ca^{2+}) and

ATPase activity measured in the presence of excess EGTA (0.5—1.0 mM). All reactions were conducted in the presence of 3 μg per ml of A23187, which effectively eliminates Ca^{2+} compartmentalization by the vesicles [1,2]. To assess monovalent cation effects on Ca^{2+}-dependent ATPase activity KCl was added at a concentration of 100 mM. The concentrations of $MgCl_2$, $CaCl_2$, and Tris-ATP added are indicated in the figure legends. An ATP regenerating system consisting of 75 μg per ml of pyruvate kinase (salt-free), 3 mM phospho*enol*-pyruvate (tricyclohexylamine salt), and 1 mM KCl was used in experiments in which ATP was added in low concentrations. KCl was required to activate the regenerating system [2], but at the low concentration of 1 mM it did not significantly change Ca^{2+}-dependent ATPase activity [1,5,6]. When ATP was used at constant millimolar concentrations, the regenerating system was omitted. Although the regenerating system stimulated ATPase activity [2], the patterns of activation by the various cations were not affected [2,12]. All reactions were linear with respect to time for incubation periods of up to 20 min. Protein was determined by the method of Lowry et al. [13].

Calculation of ionized Mg^{2+} and Ca^{2+} concentrations

The predominant chelator of ionized Mg^{2+} is ATP^{4-}. The association constant of ATP^{4-} for ionized Mg^{2+} was taken as $4.47 \cdot 10^4$ [14]. At pH 7.0 ATP^{4-} and $HATP^{3-}$ are present in approximately equimolar concentrations, but $HATP^{3-}$ does not bind a significant fraction of the ionized Mg^{2+} [14,15]. For the experiments in which ionized Mg^{2+} concentrations were calculated, the small concentration Ca^{2+} used (50 μM) did not complex with a significant fraction of the total ATP. When the concentration of ionized Ca^{2+} set with a Ca^{2+}/EGTA buffer was calculated, the association constant of Schwarzenbach et al. [16] was used, as recently described by Owen [17].

Materials

Pyruvate kinase (type III), phospho*enol*pyruvate (tricyclohexylamine salt), and Tris-ATP were obtained from the Sigma Chemical Co., St. Louis, MO. A23187 was generously supplied by Dr. R.L. Hamill, Eli Lilly and Co.

Results

ATP concentration and Ca^{2+}-dependent ATPase activity

Stimulation of Ca^{2+}-dependent ATPase activity by K^+ varied with the concentration of ATP (Fig. 1). As shown previously [2], K^+-stimulation of enzymic activity occurred at all concentrations of ATP tested, but it was greatest at millimolar levels of the nucleotide (Fig. 1, upper panel). When the total ATP concentration approached or exceeded the total Mg^{2+} concentration of 3 mM (Fig. 1, upper panel), activities decreased, whether or not K^+ was present. However, the relative decrease in activity measured with excess ATP was greatest when K^+ was not included in the incubation medium. For example, at 3 mM total Mg^{2+} and 5 mM ATP (approx. 60 μM ionized Mg^{2+}) activity measured with K^+ was still about twice the activity measured at 20 μM ATP, whereas activity measured in the absence of K^+ was decreased to 30% of the activity measured at 20 μM ATP. We have shown previously that phosphopro-

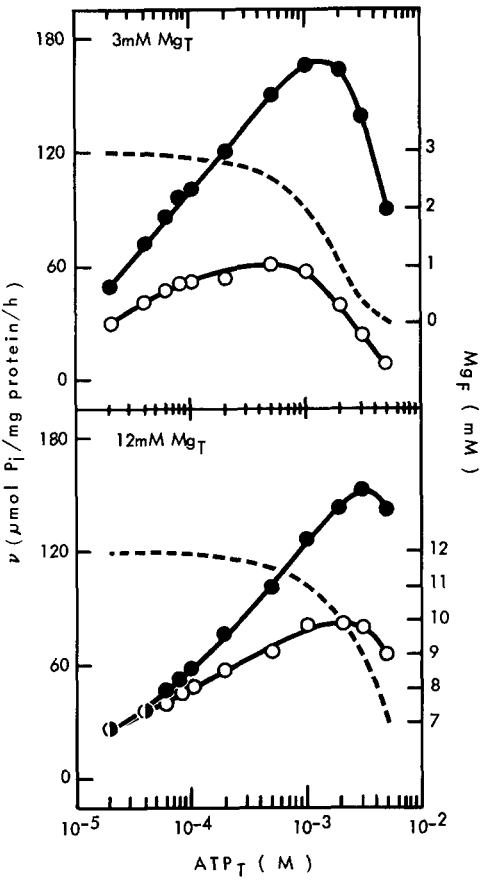

Fig. 1. ATP concentration and K^+ activation of Ca^{2+}-dependent ATPase activity (ν) at 3 mM and 12 mM Mg^{2+}. ATPase activity was measured in a medium containing 50 mM histidine (pH 7.0), 50 μM $CaCl_2$, 3 μg/ml A23187, 1 mM KCl, 3 mM phospho*enol*pyruvate, and 75 μg/ml pyruvate kinase. In the upper panel the total Mg^{2+} concentration was 3 mM, and in the lower panel it was 12 mM. Total ATP concentrations (ATP_T) were varied as indicated on the abscissa. Ionized or free magnesium concentrations (Mg_F) are indicated by the dashed lines. ○, no additional KCl; ●, 100 mM additional KCl. Ca^{2+}-independent activities were measured in the same media which also contained 1 mM Tris/EGTA. Only Ca^{2+}-dependent activities are depicted.

tein levels do not change appreciably over the range of ATP concentrations tested [2]. These results suggest that ATP activates turnover of the enzyme, but apparently there is also a requirement for ionized Mg^{2+}.

When the same concentrations of ATP were used at a total Mg^{2+} concentration of 12 mM, the ionized Mg^{2+} concentration was always 7 mM or greater (Fig. 1, lower panel). Increasing the concentration of ATP again stimulated the hydrolysis of ATP, but stimulation of activity by K^+ was attenuated. ATP concentrations of 10^{-4} M or greater were now required before K^+-stimulated ATPase activity could even be measured. With a large excess of Mg^{2+} relative to ATP, high concentrations of ATP (3—5 mM) no longer appreciably inhibited enzyme activity, which supported the conclusion that the enzyme requires ionized Mg^{2+} in addition to Mg · ATP. A summary of K^+ stimulation of Ca^{2+}-dependent ATPase activity at several total Mg^{2+} and ATP concentrations is

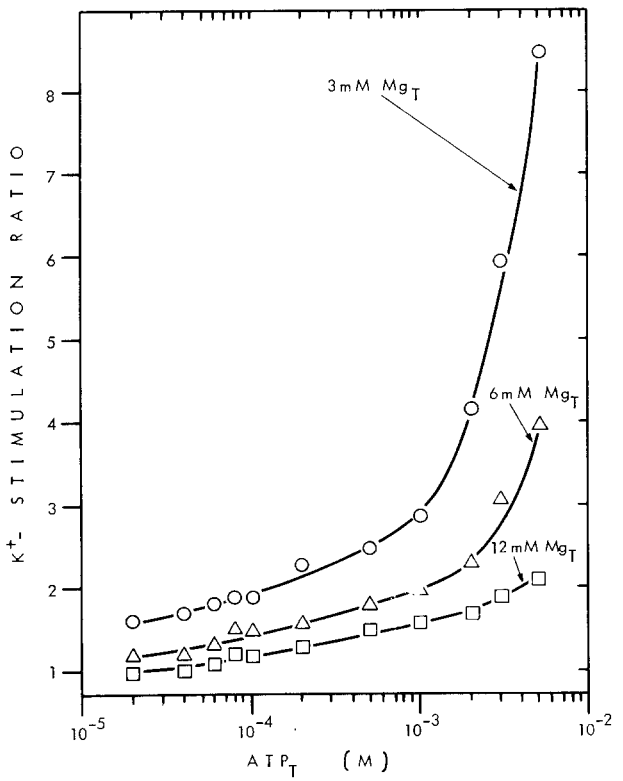

Fig. 2. K^+ activation of Ca^{2+}-dependent ATPase activity at varying total ATP and Mg^{2+} concentrations. Ca^{2+}-dependent ATPase activity measured in the presence of K^+ was divided by the same activity measured in the absence of K^+ (K^+ stimulation ratio, ordinate) for each total concentration of ATP (ATP_T). Total Mg^{2+} concentrations were 3 mM (○), 6 mM (△), and 12 mM (□). Incubations were conducted as described in Fig. 1.

depicted in Fig. 2. Stimulation of activity by K^+ varied over an 8-fold range depending upon the concentrations of Mg^{2+} and ATP used. For all concentrations of Mg^{2+} tested, stimulation by K^+ was greatest at millimolar ATP concentrations. Increasing the concentration of ionized Mg^{2+} attenuated the K^+ effect, however, even at the highest concentration of ATP tested. With an excess of 7 mM ionized Mg^{2+}, however, at 5 mM ATP (12 mM total Mg^{2+}), K^+ still increased enzymic activity 2-fold (Fig. 2, squares).

Hill plots of Mg · ATP activation of Ca^{2+}-dependent ATPase activity at 3, 6, and 12 mM total Mg^{2+} concentrations are depicted in Fig. 3. Ionized Mg^{2+} concentrations did not change appreciably over the rate of Mg · ATP concentrations tested (0.02—0.5 mM). K^+ increased the concentration of Mg · ATP required for half-maximal enzymic activation ($K_{0.5}$) by approx. 3.3-fold at each concentration of total Mg^{2+} used (Fig. 3, lower right inset). This was apparent from the shifts to the right of the lines depicting K^+ at each corresponding Mg^{2+} concentration. As the concentration of Mg^{2+} increased, the $K_{0.5}$ for Mg · ATP also increased in the presence or absence of K^+ (Fig. 3, lower right inset). Hill coefficients for Mg · ATP were less than one for all incubation conditions (Fig. 3, upper left inset). Coefficients less than one for Mg · ATP activa-

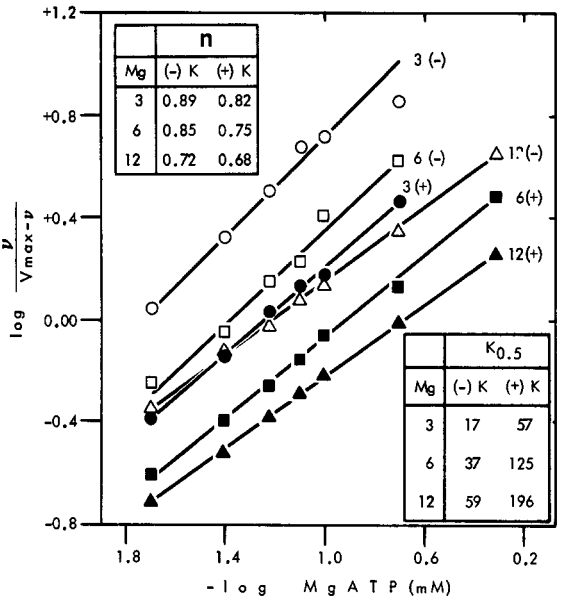

Fig. 3. Hill plots of Mg · ATP activation of Ca^{2+}-dependent ATPase activity (ν) measured in the presence and absence of K^+ at 3 mM (○,●), 6 mM (□,■), and 12 mM (△,▲) total Mg^{2+}. Incubations were conducted as described in Fig. 1. Filled and open symbols depict activities measured with (+) and without (—) 100 mM KCl, respectively. For the range of Mg · ATP concentrations plotted, the ionized Mg^{2+} concentrations did not change appreciably. Upper left inset, Hill coefficients for corresponding ATPase activities; lower right inset, Mg · ATP concentrations for half-maximal activation ($K_{0.5}$ values in μM) of the corresponding ATPase activities.

tion of the Ca^{2+}-dependent ATPase activity of skeletal muscle sarcoplasmic reticulum preparations have been obtained by several investigators [12,18,19].

Mg^{2+} concentrations and Ca^{2+}-dependent ATPase activity

The preceding results showed that K^+ stimulation of Ca^{2+}-dependent ATPase activity was optimal at millimolar Mg · ATP concentrations and at micromolar ionized Mg^{2+} concentrations. At a constant ATP concentration of 3 mM, added Mg^{2+} activated the enzyme in the presence of K^+ half-maximally at a concentration of approx. 0.5 mM (Fig. 4). The ionized Mg^{2+} concentration at this concentration of ATP was approx. 10 μM. Basal ATPase activity (measured in the presence of 1 mM EGTA) was also activated half-maximally at a similar concentration of added Mg^{2+}. In contrast, for Ca^{2+}-dependent ATPase activity measured in the absence of K^+, half-maximal activation by Mg^{2+} was increased to a level of approx. 3.0 mM. This suggested that an ionized Mg^{2+} concentration of several mM was rquired for optimal expression of Ca^{2+}-dependent ATPase activity measured without K^+. The sigmoidal shape of the curve was apparently a reflection of the change in the ionized Mg^{2+} concentration when ATP was present in excess of the total concentration of Mg^{2+}. Fig. 1 shows that Mg · ATP activated the enzyme maximally at a concentration of approx. 1 mM. Therefore, in Fig. 4 the relative low constant Ca^{2+}-dependent ATPase activity measured at 0.5—1.0 mM Mg^{2+} added in the absence of K^+ may have represented activation of the enzyme by only Mg · ATP. The additional large incre-

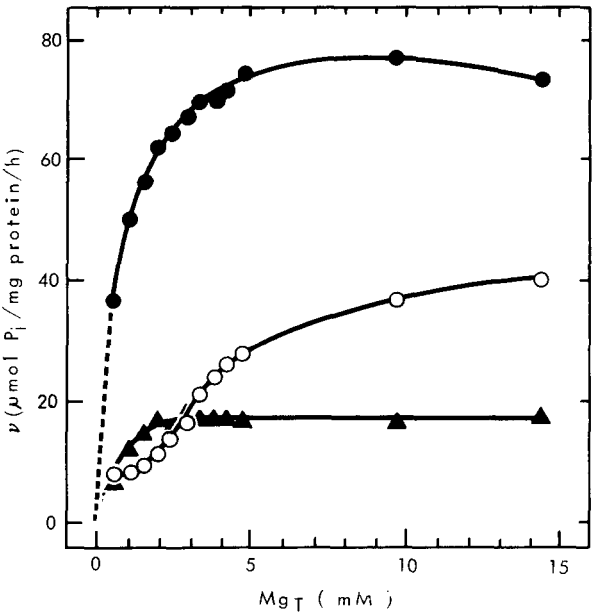

Fig. 4. Mg^{2+} concentration effects on ATPase activities (ν) at 3 mM total ATP. Incubations were conducted in the presence of 50 mM histidine (pH 7.0), 50 μM $CaCl_2$, 3 μg/ml A23187, and 3 mM Tris-ATP. The total Mg^{2+} concentration (Mg_T) was varied as indicated on the abscissa. ●, Ca^{2+}-dependent ATPase activity measured in the presence of 100 mM KCl; ○, Ca^{2+}-dependent ATPase activity measured in the absence of KCl. Ca^{2+}-independent ATPase activities were measured in the same media which in addition contained 1 mM Tris/EGTA. ▲, Ca^{2+}-independent ATPase activity measured in the presence of 1 mM Tris/EGTA and no added KCl.

ment in activity between 1.5 and 15 mM Mg^{2+} added could be attributed to binding of ionized Mg^{2+} to a second site on the enzyme. Plotting this increment in activity as a function of the ionized Mg^{2+} concentration supported this assessement (Fig. 5). The Hill plot gave a $K_{0.5}$ for ionized Mg^{2+} of 1.1 mM (or

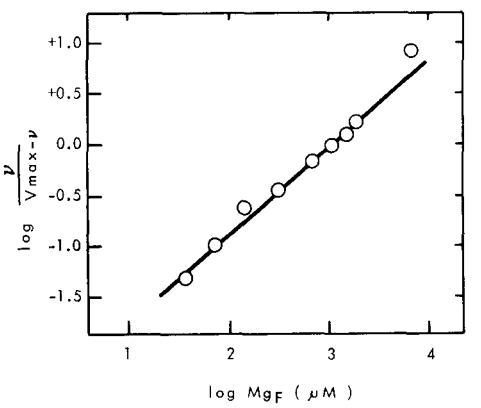

Fig. 5. Hill plot of ionized Mg^{2+} activation of Ca^{2+}-dependent ATPase activity measured in the absence of KCl. The increment in activity stimulated by total Mg^{2+} in excess of 1 mM (ν) was calculated from the data of Fig. 4. V for this activity was 32.2 μmol P_i/mg/h at a total Mg^{2+} concentration of 15 mM. The free or ionized Mg^{2+} concentration (Mg_F) corresponding to each total Mg^{2+} concentration is plotted along the abscissa.

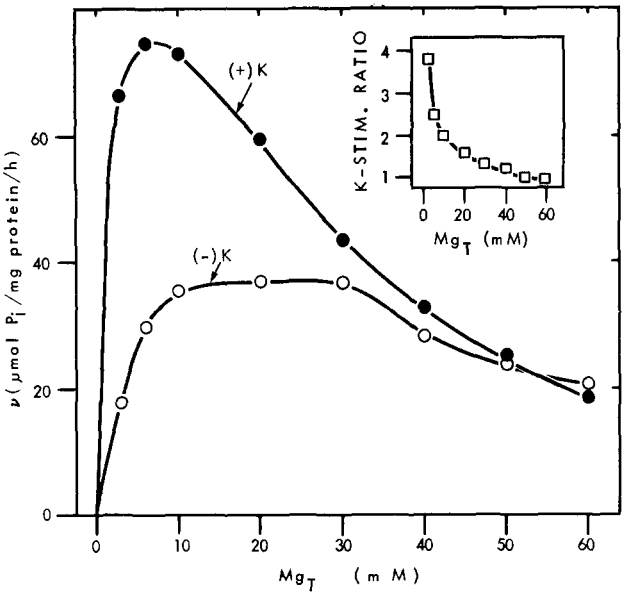

Fig. 6. Mg^{2+}-inhibition of K^+-stimulated Ca^{2+}-dependent ATPase activity (ν). The incubation medium contained 50 mM histidine (pH 7.0), 50 μM $CaCl_2$, 3 mM Tris/ATP and 3 μg/ml A23187. Total Mg^{2+} was added as indicated on the abscissa. •, Ca^{2+}-dependent activities measured in the presence of 100 mM KCl; ○, Ca^{2+}-dependent activities measured in the absence of KCl. Ca^{2+}-independent activities were measured in the same media, which also contained 1 mM Tris/EGTA. The inset depicts the ratio of K^+-stimulation for each Mg^{2+} concentration.

about 4 mM total Mg^{2+}) with a Hill coefficient of 0.9.

Even very high concentrations of ionized Mg^{2+} could not substitute for K^+ to give maximal Ca^{2+}-dependent ATPase activity (Fig. 6). In the presence of 3 mM ATP the maximal activity measured with K^+ at 3—10 mM added Mg^{2+} remained approximately twice the maximal activity which was obtained in the absence of K^+ at 10—30 mM Mg^{2+} added. High ionized Mg^{2+} concentrations (at 20—30 mM total Mg^{2+}), moreover, selectively inhibited the K^+-stimulated increment of ATPase activity, while leaving the basal Ca^{2+}-dependent ATPase activity unchanged. This provided additional evidence that the enzymic sites for K^+ were distinct from those for Mg^{2+}. At very high ionized Mg^{2+} concentrations, stimulation of activity by K^+ was completely abolished (Fig. 6, inset). In other experiments, we have shown that varying the K^+ concentration from 0.1 to 0.9 M or varying the Ca^{2+} concentration from 0.05 to 0.8 mM did not restore K^+ stimulation of the enzyme at high Mg^{2+} concentrations (unpublished observations). Thus, Mg^{2+} apparently does not inhibit K^+ stimulation of enzymic activity by competing for the putative sites on the enzyme which bind K^+.

Ca^{2+} concentration and Ca^{2+}-dependent ATPase activity

In the absence of added Ca^{2+}, Ca^{2+}-dependent ATPase activity was already nearly maximal (Fig. 7). Between 0.02 and 0.30 mM Ca^{2+} added, Ca^{2+}-dependent ATPase activity remained approximately constant when measured in the absence of K^+. Over the same Ca^{2+} concentration range, activity stimulated by K^+ was inhibited approx. 80%. At higher added Ca^{2+} concentrations, stimula-

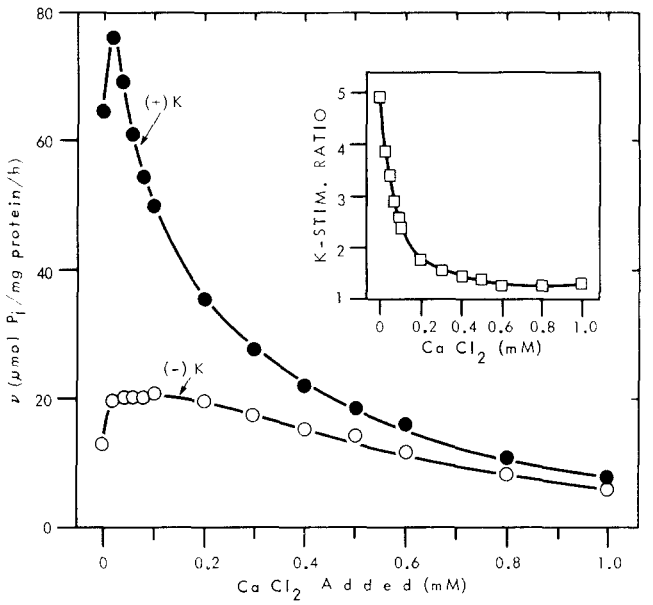

Fig. 7. Ca^{2+}-inhibition of K^+-stimulated Ca^{2+}-dependent ATPase activity (ν). The incubation medium contained 50 mM histidine (pH 7.0), 3 mM $MgCl_2$, 3 mM Tris-ATP, 3 μg/ml A23187, and the added $CaCl_2$ concentrations indicated on the abscissa. •, Ca^{2+}-dependent ATPase activity measured in the presence of 100 mM KCl; ○, Ca^{2+}-dependent ATPase activity measured in the absence of KCl. Ca^{2+}-independent activities were measured in the same media which in addition contained 1 mM Tris/EGTA and no added Ca^{2+}. The inset depicts the ratio of K^+-stimulation for each Ca^{2+}-concentration.

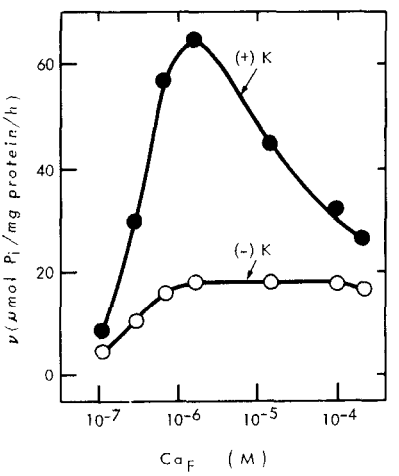

Fig. 8. K^+-stimulation of Ca^{2+}-dependent ATPase activity (ν) and ionized Ca^{2+} concentration. The incubation medium contained 50 mM histidine (pH 7.0), 3 mM $MgCl_2$, 3 mM Tris-ATP, 3 μg/ml A23187, and 0.5 mM Tris/ EGTA with (•) or without (○) 100 mM KCl. For each ionized Ca^{2+} concentration $CaCl_2$ was added in increments of 0.1 mM (0.1—0.7 mM). Ionized or free Ca^{2+} concentrations (Ca_F) are indicated on the abscissa. Only Ca^{2+}-dependent activities are plotted. Ca^{2+}-independent activities were assessed in the presence of 0.5 mM EGTA and no added Ca^{2+}. For the two highest Ca^{2+} concentrations the added Ca^{2+} concentration exceeded the EGTA concentration. Ionized Ca^{2+} is therefore overestimated somewhat for these data points because chelation of Ca^{2+} by ATP becomes significant.

tion of activity by K^+ was completely abolished (Fig. 7, inset). Thus, specific inhibition of K^+-stimulated Ca^{2+}-dependent ATPase activity could be obtained with either Mg^{2+} (Fig. 6) or Ca^{2+} (Fig. 7). Ca^{2+}/EGTA buffers were used to estimate the ionized Ca^{2+} concentration which gave maximal stimulation of enzymic activity by K^+ (Fig. 8). Maximal K^+-stimulated activity occurred at approx. 10^{-6} M ionized Ca^{2+}. At concentrations of Ca^{2+} between 10^{-5} and 10^{-4} M, K^+-activation of the enzyme was selectively inhibited. In this type of experiment it was not possible to distinguish whether it was Ca · ATP [14] or the ionized Ca^{2+} itself which actually inhibited the enzymic response to K^+.

Discussion

Monovalent cations markedly stimulate Ca^{2+} transport and the associated Ca^{2+}-dependent ATPase activity of cardiac sarcoplasmic reticulum membrane vesicles [1—4]. The same phosphoprotein enzyme intermediate of molecular weight approximately 100 000 is formed in the presence or absence of monovalent cations during the Ca^{2+} transport cycle, which suggests that only one enzyme is involved in the overall reaction mechanism [3]. The present communication provides additional evidence for distinct sites on the enzyme which can be regulated by K^+, and also characterizes some of the complex interactions between the K^+ site and the other cationic and nucleotide sites which are present on the enzyme.

K^+ effects on the activity of the cardiac enzyme are strongly dependent on the concentration of ATP used as the substrate. In a previous study, for example, it was shown that millimolar concentrations of ATP accelerated enzyme turnover by increasing the rate of decomposition of the phosphoprotein intermediate, however, this effect of ATP was greatly potentiated by K^+ [2]. The results presented presently suggest two possibilities for the synergistic action of K^+ and ATP on the rate of ATP hydrolysis. First, ATP lowers the ionized Mg^{2+} concentration by chelation, and since the enzyme active in the presence of K^+ has a lower apparent concentration requirement for ionized Mg^{2+} than the enzyme active in the absence of K^+ (Figs. 1 and 4), stimulation by K^+ is pronounced. Secondly, ATP (complexed with Mg) probably facilitates K^+ stimulation by interacting directly with the enzyme at a nucleotide binding site [2]. For example, even with a large excess concentration of free Mg^{2+} (7 mM or greater, Fig. 2) activity stimulated by K^+ is still about 2-fold, but only in the presence of 3—5 mM Mg · ATP. Hill plots showed that the concentrations of Mg · ATP required for half-maximal activity ($K_{0.5}$ values, Fig. 3) were more than 3-fold higher in the presence of K^+ than in its absence for all ionized Mg^{2+} concentrations tested. Thus it is possible that K^+ has some effect to decrease the affinity of the enzyme for Mg · ATP and that this is the reason why relatively high concentrations of Mg · ATP are required before stimulation by K^+ becomes marked. Shigekawa et al. [7] have also recently provided evidence that Mg · ATP allosterically regulates the activity of the Ca^{2+}-transport ATPase of skeletal muscle sarcoplasmic reticulum by increasing the rate of phosphoprotein hydrolysis. In that report, however, the magnitude of stimulation of activity by K^+ did not vary with the ATP concentration [7]. Ribeiro and Vianna [19] on the other hand, have shown that the enzymic sensitivity

of skeletal muscle sarcoplasmic reticulum vesicles to K^+ is increased by Mg · ATP, but only at low ionized Mg^{2+} concentrations. In a fast kinetic study Verjovski-Almeida and Inesi have very recently confirmed the allosteric interaction of ATP with the enzyme of skeletal muscle sarcoplasmic reticulum [20].

Ionized Mg^{2+} activated the Ca^{2+}-dependent ATPase activity in the absence of K^+ (Figs. 4 and 5). Even with optimal concentrations of ionized Mg^{2+}, however, the levels of activity attained never reached those which could be measured in the presence of K^+ (Fig. 6). The lower maximum velocities obtained in the presence of only Mg^{2+}, and the selective inhibition of the K^+ response by high Mg^{2+} concentrations (which was not competitive), suggests that distinct sites are present on the enzyme which can distinguish between K^+ and Mg^{2+}. In other experiments the inhibitory effect of ionized Mg^{2+} on activity assessed with K^+ was found to be a function of the Mg · ATP concentration. As the Mg · ATP concentration decreased the concentration of ionized Mg^{2+} required to inhibit activity also decreased (unpublished observations). Thus, sites on the enzyme for ionized Mg^{2+} and K^+ and Mg · ATP may all allosterically interact with one another. Consistent with this, both Mg^{2+} and K^+ increased the concentration of Mg · ATP required for half-maximal activation of ATP hydrolysis (Fig. 3). Both Shigekawa et al. [7] and Ribeiro and Vianna [19] have recently demonstrated that ionized Mg^{2+} decreases the increment of K^+ stimulation of Ca^{2+}-dependent ATP hydrolysis by skeletal muscle sarcoplasmic reticulum membrane fragments. The former investigators, moreover, also demonstrated that ionized Mg^{2+} could sometimes inhibit enzymic activity, but only in the presence of K^+. The results obtained with both cardiac and skeletal muscle preparations [7,19] therefore strongly suggest that the Ca^{2+} transport enzyme contains sites for ionized Mg^{2+} which are distinct from those for K^+.

It is well known that high ionized Ca^{2+} concentrations inhibit Ca^{2+}-dependent ATPase activity, but do not alter phosphoprotein levels appreciably [21—23]. For cardiac sarcoplasmic reticulum preparations, high ionized Ca^{2+} concentrations inhibit activity in the absence of monovalent cations, or in the presence of either K^+ or Na^+ [3]. At equimolar concentrations of Mg^{2+} and ATP the sensitivity of the enzyme to Ca^{2+} inhibition is markedly increased by monovalent cations. This can be demonstrated by either adding Ca^{2+} directly to the assay medium (Fig. 7) or by buffering the ionized Ca^{2+} concentration by adding EGTA (Fig. 8). The threshold concentration of ionized Ca^{2+} required for inhibition of activity is about 10 μM. At a higher range of Ca^{2+} concentrations inhibition of monovalent cation sensitve activity is nearly complete, whereas activity measured in the absence of K^+ is not altered appreciably. It is possible that the enzyme may exist in two main conformational states of activity defined by the presence or absence of K^+, and that the former state is more highly sensitive to Ca^{2+} inhibition. Two conformations of the forward running pump that hydrolyse ATP have recently been postulated by Shigekawa and Dougherty [24]. These investigators observed that ADP could dephosphorylate the skeletal muscle enzyme only in the presence of K^+, even though the phosphoprotein intermediate and hydrolysis of ATP could be meausred in its absence. Whether ionized Ca^{2+} itself or Ca · ATP [14] is the inhibitory agent of the cardiac enzymic activity measured with K^+ is not clear at present. Ca · ATP could conceivably inhibit the enzyme by competing with Mg · ATP for binding

to the regulatory site on the enzyme. Since Mg · ATP at this site is required for K^+ to stimulate the rate of dephosphorylation of the enzyme phosphoprotein intermediate maximally [2], competition of Ca · ATP for the regulatory site could explain why Ca^{2+} inhibition is most marked in the presence of K^+. Alternatively, ionized Ca^{2+} could bind directly to the low affinity Ca^{2+} site which is present on the enzyme [25], and thereby perhaps inhibit the response to K^+ selectively. The apparent concentration of Ca^{2+} required to saturate the low affinity site is in millimolar range [25], however, which is much greater than the concentration of Ca^{2+} required for inhibition of activity reported presently. K^+ stimulation of the Ca^{2+}-dependent ATPase activity of cardiac sarcoplasmic reticulum was greatest at an ionized Ca^{2+} concentration of about 1 μM. At higher or lower Ca^{2+} concentrations the increment of stimulation by K^+ decreased (Fig. 8). These results suggest that in intact cardiac cells K^+ regulation of Ca^{2+} transport may become maximal at a free Ca^{2+} concentration which occurs during development of tension.

In summary, the results of this study and others [1—7,19] suggest that the Ca^{2+}-transport ATPase of either cardiac or skeletal muscle sarcoplasmic reticulum is regulated allosterically. Agents that control activity by accelerating the rate of hydrolysis of the phosphoprotein intermediate include K^+ [2—4,6,7], Mg^{2+} [23,26], and Mg · ATP [2,7,20]. Distinct sites on the enzyme are probably present for each of these activators and the sites apparently interact with one another to ultimately control hydrolytic activity at the catalytic site on the enzyme. For the Ca^{2+}-dependent ATPase activity of the cardiac sarcoplasmic reticulum, Mg^{2+}, Ca^{2+}, and Mg · ATP can all regulate the activity stimulated by K^+ depending on the relative concentrations of the various effectors.

Acknowledgements

I thank Lee Lynch for her invaluable technical assistance. The excellent secretarial and editorial assistance of Kristi Wolfe is gratefully acknowledged. This work was supported in part by grants HL-06308, HL-07182, and HL-18795 from the National Heart, Lung and Blood Institute of the National Institutes of Health; by the American Heart Association, Indiana Affiliate; and by the Herman C. Krannert Fund. A preliminary report of this work was presented at the 62nd annual Meeting of the Federation of the American Socieites for Experimental Biology in April 1978 in Atlantic City, NJ [27]. The preceding paper of this series is Ref. 2.

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