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# STIMULATION OF ADENOSINE TRIPHOSPHATASE ACTIVITY OF SARCOPLASMIC RETICULUM BY ADENYLYL METHYLENE DIPHOSPHATE

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## Summary

The effects of adenylyl methylene diphosphate (AMD), a non-hydrolyzable ATP analogue, were examined in sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle. The $Ca^{2+}$-dependent ATPase activity measured at 5°C and pH 7.0 in 5.2 $\mu$M [$\gamma$-$^{32}$P]ATP and in the absence of added alkali metal salts was stimulated by added AMD. The steady state level of phosphoenzyme, however, was not decreased greatly by added AMP under these conditions. The hydrolysis of the phosphoenzyme formed at the steady state in the absence of added alkali metal salts was accelerated by added AMD to an extent that can account for the stimulation of the ATPase activity. At 5°C and pH 7.0 the maximum stimulation of phosphoenzyme hydrolysis by AMD and the $K_m$ value for this ATP analogue were 4.3-fold and 40 $\mu$M, respectively. These results provide further support for our previous conclusion (Shigekawa, M., Dougherty, J.P. and Katz, A.M. (1978) J. Biol. Chem. 253, 1442—1450) that 2 classes of ATP site exist in the calcium pump ATPase in the absence of added alkali metal salts, one being the catalytic site and the other being the regulatory site which activates the activity of the catalytic site.

## Introduction

Active calcium transport by sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle is energized by ATP hydrolysis that is catalyzed by a membrane-bound $Ca^{2+}$- and $Mg^{2+}$-dependent ATPase [1—3]. The rates of cal-

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Abbreviations: AMD, adenylyl methylene diphosphate; EGTA, ethyleneglycolbis-($\beta$-aminoethylether)-*N,N'*-tetraacetic acid.

cium transport and the concomitant $Ca^{2+}$-dependent ATP hydrolysis by these vesicles are much slower in the absence of added alkali metal salts than in the presence of optimal concentrations of these salts [4—7]. As is the case in high KCl concentrations [8—11], the terminal phosphate of ATP is transferred to the calcium pump ATPase of sarcoplasmic reticulum during ATP hydrolysis in the absence of added alkali metal salts to form an acid-stable phosphoenzyme intermediate [5,7,12—14]. The kinetic properties of the steady state phosphoenzyme formed in high $Mg^{2+}$ concentrations (1—2 mM) and low $Ca^{2+}$ concentrations (10—20 $\mu$M) but in the absence of added alkali metal salts differ significantly from those of the corresponding phosphoenzyme formed in high KCl concentrations [7,12,13]. The former does not donate its phosphate group to added ADP, whereas the major fraction of the latter donates its phosphate group to added ADP to form ATP. Either KCl or ATP accelerates the hydrolysis of the phosphoenzyme formed in the absence of alkali metal salts [12,13]. However, the role of ATP in stimulating the hydrolysis rate of the phosphoenzyme in KCl has been controversial [14—18].

It was reported previously that double reciprocal plots of $Ca^{2+}$-dependent ATPase activity vs. ATP concentration are non-linear in the absence of added alkali metal salts [7]. This finding was interpreted to suggest that the calcium pump ATPase of the sarcoplasmic reticulum has two classes of ATP-binding site in the absence of added alkali metal salts [7]. The high-affinity site ($K_m \approx$ 0.1 $\mu$M) appears to represent the phosphorylation site as the $K_m$ value for ATP is the same as that for the steady state level of phosphoenzyme. The ATP binding to the low-affinity site ($K_m \approx 30$ $\mu$M) appears to accelerate phosphoenzyme hydrolysis because increasing ATP concentrations above those which saturate the phosphorylation site increase the rate of ATP hydrolysis without affecting significantly steady state phosphoenzyme levels [7]. This conclusion is supported by the finding that the rates of phosphoenzyme hydrolysis measured directly under similar conditions are stimulated by added ATP in a high concentration range similar to that for the low affinity site identified in the kinetic study of the ATPase activity [13].

In the present study it is shown that adenylyl methylene diphosphate (AMD), a non-metabolizable ATP analogue, like high ATP concentrations, can accelerate hydrolysis of the phosphoenzyme formed in the absence of added alkali metal salts. Thus, AMD can stimulate the steady state rate of ATP hydrolysis in the presence of a relatively low concentration of ATP. The results indicate that the activity of the calcium pump ATPase of sarcoplasmic reticulum can be regulated by ATP at an 'allosteric' regulatory site which is distinct from the catalytic site.

## Materials and Methods

Sarcoplasmic reticulum vesicles, prepared from rabbit white skeletal muscle as described previously [5], were further treated with diethyl ether according to the method of Inesi et al. [19] as reported previously [5]. The diethyl ether-treated vesicles no longer accumulated $Ca^{2+}$ while the ATPase activity remained unimpaired.

Alkali metal salts were removed from the vesicles as described previously

[5]. Protein concentration, ATPase activity and phosphoenzyme level were assayed as described previously [7]. Hydrolysis of AMD was examined by incubating 0.131 mg/ml of the ether-treated vesicles in 15 mM imidazole/HCl (pH 7.0), 100 mM KCl, 2.7 mM $MgCl_2$, 27 $\mu$M $CaCl_2$ and 1.0 mM AMD for 2 min at 25°C. $P_i$ liberation from AMD could not be detected under these conditions.

Disodium ATP and dilithium AMD obtained from Boehringer Mannheim were converted to Tris-forms by passage through a cation exchange resin [5]. EGTA and Tris were purchased from Sigma Chemical Co. [$\gamma$-$^{32}$P]ATP tetra-(triethylammonium) salt (25—30 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc. All reagents were of analytical grade.

## Results and Discussion

AMD stimulated the $Ca^{2+}$-dependent ATPase activity of sarcoplasmic reticulum vesicles measured in the absence of added alkali metal salts and in the presence of 5.2 $\mu$M [$\gamma$-$^{32}$P]ATP (Fig. 1). The ATPase activity increased with increasing concentrations of AMD and reached a plateau at AMD concentrations above approx. 0.1 mM. In contrast, phosphoenzyme levels decreased slightly with increasing AMD concentrations (Fig. 1). These findings suggest that AMD increased the ATPase activity by accelerating decomposition of the phosphoenzyme intermediate. These findings also indicate that AMD did not inhibit greatly the phosphorylation of the ATPase by [$\gamma$-$^{32}$P]ATP when the non-hydrolyzable ATP analogue was present in a 50-fold excess over ATP. The catalytic site of the calcium pump ATPase thus appears to have a much lower affinity for AMD than for ATP under these conditions.

The effects of AMD on phosphoenzyme decomposition were examined after a steady state level was reached in 1.5 $\mu$M [$\gamma$-$^{32}$P]ATP in the absence of added alkali metals salts and further phosphorylation prevented by addition of excess EGTA. Addition of AMD to the reaction medium accelerated phosphoenzyme decompostion (Fig. 2). Similarly, addition of ATP also accelerated phosphoenzyme decomposition in agreement with previous results [12,13]. In either case the phosphoenzyme decomposition was accompanied by liberation of an almost stoichiometric amount of $P_i$ (Fig. 2). The phosphoenzyme decomposition observed after addition of AMD obeyed first-order kinetics (Fig. 3). The apparent first order rate constants ($k_d$) estimated for the phosphoenzyme decomposition after addition of various concentrations of AMD are plotted as a function of AMD concentration in Fig. 4. As shown in the figure the apparent rate constant increased with increasing AMD concentration and exhibited saturation at high AMD concentrations (>0.22 mM).

It was found previously [7] that the ratios of $Ca^{2+}$-dependent ATPase activities to phosphoenzyme levels at the steady state, obtained in the absence of alkali metal salts and under conditions similar to those described for Fig. 4, are constant at ATP concentrations between 0.026 and approx. 1 $\mu$M. Increasing ATP concentration in the range above 1 $\mu$M stimulated ATPase activity without increasing the steady state level of phosphoenzyme. These findings suggest that ATP accelerates phosphoenzymes decomposition only at ATP concentrations above approx. 1 $\mu$M. Thus, the AMD-dependent increment of the apparent rate

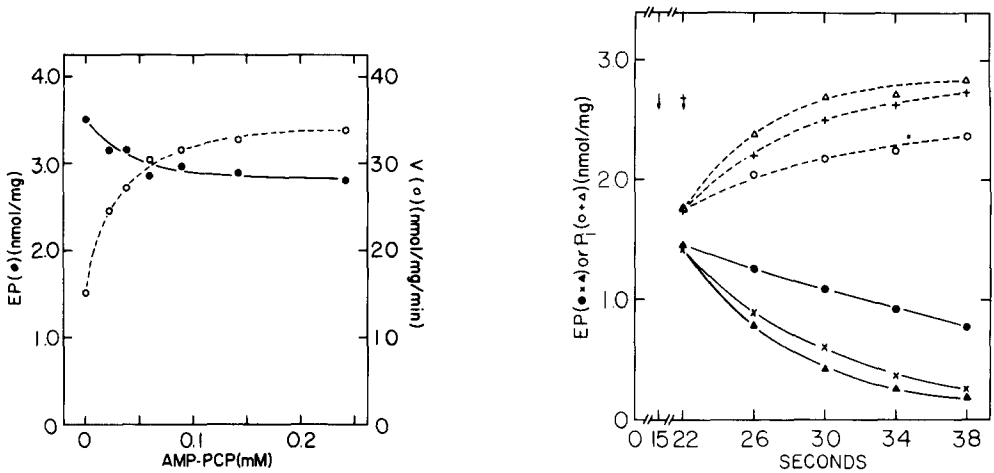


Fig. 1. Effects of AMD on ATPase activity and phosphoenzyme level in the absence of added alkali metal salts. Reactions were carried out at 5°C for 15 s with 0.177 mg/ml diethyl ether-treated vesicles in 15 mM imidazole-HCl (pH 7.0), 20 μM $CaCl_2$, 5.2 μM [$\gamma$-$^{32}$P]ATP, and various concentrations of AMD and $MgCl_2$. The latter was present at a concentration that is 2 mM in excess of that of AMD. In this experiment AMD was added to the reaction medium immediately before addition of [$\gamma$-$^{32}$P]ATP started the ATPase reaction.

Fig. 2. Effects of AMD and ATP on the decomposition of the phosphoenzyme formed in the absence of added alkali metal salts. The diethyl ether-treated vesicles, 0.204 mg/ml, were phosphorylated at 5°C in 15 mM imidazole-HCl (pH 7.0), 2.7 mM $MgCl_2$, 10 μM $CaCl_2$ and 2.0 μM [$\gamma$-$^{32}$P]ATP. 15 s after the start of the reaction (↓), 0.05 ml 150 mM EGTA was added to 1.0 ml of the reaction medium to prevent further phosphorylation. At 22 s (‡), 0.05 ml $H_2O$ (○, ●), a mixture of 2.17 mM AMD and 2.2 mM $MgCl_2$ (+, X), or a mixture of 4.3 mM ATP and 4.4 mM $MgCl_2$ (△, ▲) were added to the reaction medium, and phosphoenzyme decomposition and $P_i$ liberation were followed.

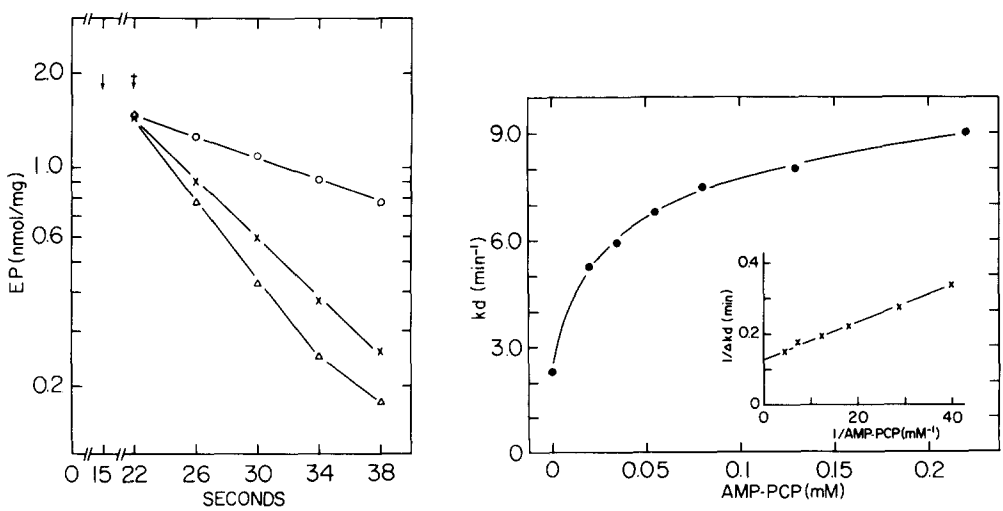


Fig. 3. Semilogarithmic plot of phosphoenzyme level against time after addition of AMD or ATP. Data are those in the experiment shown in Fig. 2.

Fig. 4. Dependence on added AMD concentrations of the apparent rate constants of the phosphoenzyme decomposition obtained in the absence of added alkali metal salts. The apparent rate constants were estimated from the slope of the linear semilogarithmic plot of the phosphoenzyme levels against time after addition of various concentrations of AMD as described in Fig. 3. Experimental conditions were the same as those described in the legend to Fig. 2 except that initial [$\gamma$-$^{32}$P]ATP concentration was 1.3 μM and the diethyl ether-treated vesicles were present at a concentration of 0.20 mg per ml, respectively. $MgCl_2$ concentrations were by 2.43 mM in excess over AMD added to the reaction medium. Because of the hydrolysis of ATP during initial 22 s, ATP concentration in the medium was 0.65 μM at the time of AMD addition.

constant ($\Delta k_d$) for phosphoenzyme decomposition was estimated by subtracting the value of the rate constant obtained in 0.65 $\mu$M ATP from that obtained at each AMD concentration. As shown in the inset to Fig. 4, the double reciprocal plot of the AMD-dependent increment of the apparent rate constant ($\Delta k_d$) vs. AMD concentration was linear, suggesting that 1 mol of AMD per mol of phosphoenzyme is involved in the stimulation of the rate of phosphoenzyme hydrolysis. As excess $MgCl_2$ (more than 2.43 mM) is present in the reaction medium and the association constant of AMD with $Mg^{2+}$ was reported to be $1.29 \cdot 10^4$ $M^{-1}$ at pH 7.4 [20], AMD mainly exists as a Mg-AMD complex under the experimental conditions of Fig. 4. The $K_m$ value for AMD estimated from the linear plot shown in the inset to Fig. 4, was 40 $\mu$M and the observed maximal stimulation was 4.3-fold. These values are similar to those obtained previously for MgATP under similar experimental conditions ($K_m$ = 35 $\mu$M: maximal stimulation, 4.6-fold) [13]. These observations thus indicate that AMD, which is not hydrolyzed by the calcium pump ATPase of the sarcoplasmic reticulum, can effectively substitute for ATP in accelerating the hydrolysis of the phosphoenzyme intermediate. In this context, it is interesting to note that ADP did not accelerate phosphoenzyme hydrolysis under similar conditions [13].

The present findings, together with our previous observations [7,12,13] provide strong evidence that two classes of ATP site exist in the calcium pump ATPase. That with the high affinity for ATP is the catalytic site. The other, which binds ATP only at higher concentrations, is a regulatory site that, when bound to ATP or AMD activates the catalytic site. Both sites appear to coexist in the normal reaction of the calcium pump ATPase in the absence of added alkali metal salts.

It was reported previously that non-linear double reciprocal plot of the ATPase activity vs. ATP concentration can also be observed in the presence of high (approx. 100 mM) KCl concentrations [15,19,21—23,25]. The steady state levels of phosphoenzyme intermediate also increase with increasing ATP concentrations under these conditions [15]. Because hydrolysis of the phosphoenzyme intermediate has been considered to be the rate-limiting step in the overall rate of ATP hydrolysis in the presence of high KCl concentrations [15,16] and high concentrations of added ATP did not appear able to accelerate phosphoenzyme hydrolysis under these conditions [15,16], Kanazawa et al [15] concluded that the substrate stimulation of the ATPase activity observed in the presence of high KCl concentrations is due to acceleration of formation of phosphoenzyme intermediate by high concentrations of ATP.

The non-linear double reciprocal plot of the ATPase activity versus ATP concentration in high KCl concentrations [19,21—23,25] has been interpreted in terms of negative cooperative interaction between the catalytic sites for ATP or in terms of the existence of two classes of ATP-binding site on the ATPase enzyme. The recent finding by Eckert et al. [24] that an ATP concentration range similar to that for the low affinity site identified in the kinetic study of the ATPase activity in the presence of high KCl concentrations protected against inhibition of the ATPase activity by a sulfhydryl reagent, appears to be compatible with the latter interpretation. Dupont [25] has recently observed

that high concentrations of pyrophosphate or $\alpha,\beta$-methylene adenosine 5′-triphosphate (AMP-($CH_2$)*PP*) which are not hydrolyzed or are hydrolyzed slowly by the calcium pump ATPase, respectively, could stimulate the rate of ATP hydrolysis by sacoplasmic reticulum vesicles in the presence of high KCl concentrations. These findings thus suggest that in the ATPase activity in high KCl concentrations as in the absence of added alkali metal salts, ATP acts at both high- and low-affinity sites, the latter being the regulatory site that regulates the catalytic activity of the former.

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