Product Inhibition of the Actomyosin Subfragment-1 ATPase in Skeletal, Cardiac, and Smooth Muscle

Jean S. Drew, Vijay A. Harwalkar, and Leonard A. Stein

We studied product inhibition of the actin-activated ATPase of myosin subfragment-1 (S-1) from the three types of muscle tissue: skeletal, cardiac, and smooth. Increasing levels of [MgADP] in the 0–1-mM range caused significant inhibition of the actin-activated MgATPase activity of cardiac and gizzard but not skeletal muscle S-1. When total nucleotide concentration ([ATP] + [ADP]) was kept constant at 1 mM, ATPase activity was inhibited by 50% at an ADP/ATP ratio of 6:1 for cardiac S-1 and 3:1 for gizzard S-1. For skeletal S-1, however, even a 19:1 ratio did not cause 50% inhibition of ATPase activity. The observed effect was not due to changes in pH or inorganic phosphate concentration, nor could it be explained by substrate (ATP) depletion. In the absence of actin, ADP had little or no inhibitory effect on the ATPase activity of S-1, and these observations imply that ADP is competing directly for the ATP binding site of the actin–S-1 complexes of cardiac and smooth muscle S-1. ADP has previously been shown to be a weak competitive inhibitor of the ATPase activity in skeletal muscle. The current data imply that ADP is a very effective competitive inhibitor for the actin-activated ATPase activity of cardiac and gizzard S-1 and, therefore, that ADP may be a physiologically important modulator of contractile activity in cardiac and smooth muscle. (Circulation Research 1992;71:1067–1077)

KEY WORDS • ADP • subfragment-1 • ATPase • gizzard • cardiac muscle

Contraction of both striated and smooth muscle results from the cyclic interaction of myosin and actin. This interaction is driven by the hydrolysis of ATP to ADP and inorganic phosphate (Pi) by the myosin ATPase. To gain an understanding of the actin–myosin interaction from a molecular standpoint, biochemists have turned to the biochemical kinetics of the actin-activated myosin ATPase activity, which is the intrinsically correlated to contraction in vivo. Kinetic studies of the myosin ATPase activity of striated myosin have led to the development of a six-state kinetic model capable of accounting for the great majority of the available pre-steady-state and steady-state data1 (see Figure 1a), and recent physiological data also appear to support this model.2–5 In the six-state model, when actomyosin subfragment-1 (acto-S-1) is added to ATP, a rapid equilibrium (equilibrium binding constant K3, Figure 1a) is established between actin-bound and actin-free subfragment-1 (S-1)–ATP complexes (i.e., M-ATP, where M is S-1; Figure 1a). Hydrolysis of ATP then occurs on the surface of the myosin molecule in both the actin-free and actin-bound pathways (see Figure 1a, M-ATP ↔ M-ADP-Pi, and A-M-ATP ↔ A-M-ADP-Pi, respectively, where A is actin), and the rate of this “rapid” hydrolysis is very fast compared with the steady-state ATPase rate. After this rapid hydrolysis, a conformational change occurs within the myosin–ATP complex (M-ADP-Pi ↔ M-ADP-Pi, and A-MADP-Pi, ↔ A-M-ADP-Pi, and this transition is rate limiting to steady-state hydrolysis. After this conformational change, the products of hydrolysis, ADP and Pi, are released from the myosin surface, and the cycle begins again in the presence of ATP. In this model, actin activates the ATPase activity by binding to the M-ADP-Pi complex and by increasing the rate of product release (i.e., product release is much slower in the absence of actin).

To simplify the mathematical modeling of the six-state model, it is usually assumed that the “on” rate of ATP to acto-S-1 is very rapid and that the “off” rate for the products of hydrolysis is not rate limiting for hydrolysis. Hence, there is no significant population of the states A-M or A-M-ADP during the steady-state hydrolysis of ATP. However, routine studies in our laboratory on the steady-state hydrolysis of ATP by acto-S-1 of cardiac and smooth muscle origin show that the actin-activated ATPase activity decreases with time as the reaction progresses. This is not due to the protein denaturing, because addition of ATP returns the rate toward normal. Skeletal S-1 shows a fall in rate when the substrate is virtually exhausted, but in the cardiac and smooth muscle systems, the rate falls at ATP concentrations normally considered to be saturating.

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These findings suggest that product inhibition is responsible for the fall in ATPase activity observed for cardiac and smooth muscle acto-S-1, and this was the impetus for the present studies. One possible model for product inhibition is that ADP is competing directly with ATP for the ATP binding site. Because of the data presented herewith, we favor this model as shown in Figure 1b.

Recently, there have been several reports of the effects of ADP on the acto–myosin interaction. In prior solution studies, ADP appeared to be a weak competitive inhibitor of the skeletal acto-S-1 MgATPase. Inhibition by ADP has also been reported for thiophosphorylated gizzard myosin using an in vitro motility assay. In cardiac muscle in vivo, hypoxic ventilation is accompanied by severe depression of cardiac function as well as a 50% increase in cardiac tissue ADP level. In smooth muscle cells in vitro, ADP increases tension development and slows down crossbridge detachment. Also, the local concentration of ADP at the contractile apparatus may rise significantly during contraction in vivo. These findings suggest that ADP inhibition may be a physiologically important modulator of ATPase activity in cardiac and smooth muscle tissues. Prior kinetic studies on the rate of ADP release from bovine myosin acto-S-1 found that ADP release was an order of magnitude slower in cardiac than in skeletal acto-S-1. However, the “off” rate measured was still one to two orders of magnitude greater than the $V_{\text{max}}$ of the steady-state ATPase activity and, therefore, was not rate limiting for the ATPase cycle.

The purpose of the present study was to evaluate ADP inhibition of the actin-activated ATPase of isolated myosin S-1 from the three distinct muscle tissues: cardiac, skeletal, and smooth. Smooth muscle S-1 has traditionally been neglected as a kinetic model for smooth muscle myosin. However, we have observed recently that unphosphorylated gizzard S-1 has an actin-activated $V_{\text{max}}$ similar to that of phosphorylated heavy meromyosin (HMM) and an MgATPase rate in the absence of actin that is comparable to that of phosphorylated HMM in the extended conformation. Thus, we believe that S-1 can indeed be a useful model, along with HMM, for investigating actin–myosin interactions in smooth muscle.

Materials and Methods

Proteins

Actin and skeletal myosin were prepared from rabbit skeletal muscle, and cardiac myosin was purified from swine cardiac tissue. Smooth muscle myosin was isolated from frozen turkey gizzards. S-1 from skeletal and cardiac myosin was obtained by chymotryptic digestion and from gizzard myosin by papain digestion. We have also prepared gizzard S-1 by digestion with Staphylococcus aureus protease. The two gizzard subfragments differ in that the 20-kd regulatory light chain (LC20) and the 26-kd S-1 heavy chain fragment were cleaved by papain but not $S.\text{aureus}$ protease, and this cleavage was associated with threefold to fourfold strengthened actin affinity for papain-digested S-1. However, there were no differences in the effects of ADP on papain versus $S.\text{aureus}$ protease–digested S-1 kinetics. In the present study, we report the results for papain-digested S-1.

Chymotrypsin was obtained from Worthington Biochemical Corp., Freehold, N.J., and papain was obtained from Sigma Chemical Co., St. Louis, Mo.

Kinetic Studies

ATPase assays were performed as described previously by ($\gamma$-32P)ATP hydrolysis assay. ATPase data were analyzed by a hyperbolic least-squares fitting procedure. For the inhibition assays, mathematical analysis of Figure 1b predicted a simple hyperbolic dependence of ATPase activity on [ATP], if the experiments were performed with the sum of [ADP] and [ATP] kept constant. Thus, instead of performing experiments at several fixed levels of [ADP] and varying [ATP], we varied both [ADP] and [ATP] from 0 to 1 mM, such that the total nucleotide concentration, [ATP] + [ADP], was kept constant at 1 mM and the ionic strength was kept constant at 13 mM. In all experiments for which [ADP] and [ATP] were varied, the reported concentrations were corrected for ATP split during the experiment. It is of note that the data obtained in this way will be applicable to the determination of the model in Figure 1b, once techniques are devised to measure kinetic rate.
TABLE I. Steady-State Actin-Activated Mg\textsuperscript{2+} ATPase Activity of Skeletal, Cardiac, and Gizzard Subfragment-1

<table>
<thead>
<tr>
<th>Muscle type</th>
<th>(V_{\text{max}}) (sec\textsuperscript{-1})</th>
<th>(K_{\text{ATPase}}) ((\mu\text{M actin}))</th>
<th>(K_{\text{binding}}) ((\mu\text{M actin}))</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal</td>
<td>3.33</td>
<td>2.7</td>
<td>11.6</td>
<td>15</td>
</tr>
<tr>
<td>Cardiac</td>
<td>1.74</td>
<td>5.1</td>
<td>26.0</td>
<td>15</td>
</tr>
<tr>
<td>Smooth</td>
<td>1.40</td>
<td>10.8</td>
<td>26.5</td>
<td>25</td>
</tr>
</tbody>
</table>

\(V_{\text{max}}\), maximum velocity; \(K_{\text{ATPase}}\), activation constant of actin-activated ATPase activity; \(K_{\text{binding}}\), dissociation constant of subfragment-1 to actin.

Conditions were as follows: 10 mM imidazole (pH 7.0), 1 mM MgCl\textsubscript{2} (free), 0.5 or 1 mM MgATP, and 1 mM dithiothreitol; ionic strength maintained at 13 mM with added 2.5 mM KCl when necessary.

constant \(k_{\text{ATP}}\) directly and accurately (see Figure 1b), and this will be the subject of a subsequent report.

Mathematical Analysis of Inhibition Data

The data presented are shown to fit hyperbolic kinetics quite well, and it is of interest to use the hyperbolic fitting parameters to determine the ratio of \([\text{ADP}] / [\text{ATP}]\) that gives 50% inhibition of the ATPase, \([\text{ADP}] / [\text{ATP}]\)\textsubscript{50%}. If it is assumed that the total nucleotide concentration, \([\text{ADP}] + [\text{ATP}]\), is 1 mM, our model predicts that the hyperbolic dependence of ATPase activity on [ATP] can be written as

\[1/V_{\text{ATP}} = F_1 + F_2/[\text{ATP}]\]  (1)

where \(V_{\text{ATP}}\) is the ATPase rate and \(F_1\) and \(F_2\) depend on the kinetic parameters in Figure 1, the ratio of the rapid equilibrium binding constants \(K_{\text{ADP}}/K_{\text{ATP}}\), and the kinetic rate constants \(k_{\text{ADP}}\) and \(k_{\text{ATP}}\). \(F_1\) and \(F_2\) are experimentally determined from the data using a least-squares fitting procedure based on Nelder and Mead.\textsuperscript{21}

In our system, the maximal ATPase rate occurs, by definition, at [ATP]=1 mM and [ADP]=0. Substituting 1 for [ATP] in Equation 1, we find the maximum rate to be given by

\[V_{1 \text{mM ATP}} = 1/(F_1 + F_2)\]

Hence, the [ATP] that gives half the maximum rate can be determined from

\[V_{1/2} = V_{1 \text{mM ATP}}/2 = 1/[2(F_1 + F_2)] = [\text{ATP}]/(F_2 + F_1[\text{ATP}])\]

At 50% inhibition we find that

\[[\text{ADP}] / [\text{ATP}]\]\textsubscript{50%} = (F_1 + F_2)/F_2  \quad (2)

Another way to view the system is as a simple competitive inhibition system with the phenomenological equation

\[V = V_{\text{max}}[S]/K_m (1 + [I]/K_i) + [S]\]  (3)

where \(V\) is the observed ATPase rate, \(V_{\text{max}}\) is the rate extrapolated to infinite [ATP], [S] is [ATP], [I] is [ADP], and \(K_m\) is the apparent activation constant of the ATPase activity for ATP, and \(K_i\) is the apparent inhibition constant for ADP. If we then require that \([\text{ATP}] + [\text{ADP}] = 1\) and assign \(F_1\) and \(F_2\) analogously to Equation 1, i.e., if \(F_1 = (1 - K_m/K_i)\) \(V_{\text{max}}\) and \(F_2 = (K_m + K_m/K_i)/V_{\text{max}}\), we find that

\[K_m / K_i = F_2/[F_2 + F_1] + F_1 Km/[F_2 + F_1] \quad (4)\]

If \(K_m\) can be assumed to be very small (i.e., less than 0.01 mM), then \(K_m / K_i\) is approximately equal to \(([\text{ADP}] / [\text{ATP}]\)\textsubscript{50%}. Note that the ratio \(K_m / K_i\) is not the ratio of the ADP and ATP binding constants \(K_{\text{ADP}}/K_{\text{ATP}}\) but is related to this ratio in a complex manner. The relation involves \(k_{\text{ATP}}\) and \(k_{\text{ADP}}\), the ATP and ADP “off” rates, respectively, as well as all the other rate constants in the model given in Figure 1b.

Results

Kinetic Characterization of Skeletal, Cardiac, and Smooth Muscle Proteins

In Table 1, the kinetic constants \(V_{\text{max}}\), \(K_{\text{ATPase}}\) (the activation constant of actin-activated ATPase activity), and \(K_{\text{binding}}\) (the dissociation constant of S-1 to actin) for the skeletal, smooth, and cardiac muscle preparations used are listed. The constants given for skeletal and cardiac muscle are in agreement with previously reported values from this laboratory, whereas the values for smooth muscle acto-S-1 are the first to be reported from this laboratory.

Inhibition of Actin-Activated ATPase Activity During Hydrolysis Over Time

Figure 2 shows the progressive inhibition of the actin-activated ATPase of cardiac S-1 as the hydrolysis of 1 mM ATP goes to completion. The ATPase activity was reduced by 20–30% of its initial value when 50% of the ATP had been hydrolyzed and by 50% when 80% of the ATP had been hydrolyzed. A concentration of 0.2 mM ATP would be near saturating for cardiac S-1 in the absence of added ADP,\textsuperscript{22} yet significant inhibition of ATPase activity was observed. The observed inhibition was unlikely to be secondary to a change in pH, since only a small drop in pH was observed in these experiments (approximately a 0.2-pH drop after 1 mM ATP was hydrolyzed to completion). However, measurement of pH in the presence of relatively concentrated protein solutions is difficult. Hence, we decided to control pH directly by hydrolyzing less than 0.1 mM ATP in each experiment, using the time course of complete hydrolysis of 1 mM ATP (Figure 2) as a control value.

ADP Inhibition of Skeletal, Cardiac, and Gizzard S-1 ATPases: Eliminating the Effect of Variable pH and Ionic Strength

The next set of experiments was designed to mimic the presumed changes in [ADP] over time, as seen in Figure 2, by individually controlling [ADP] and [ATP]. Since we wished to compare all the experiments performed in a quantitative fashion, it was mandatory to keep the ionic strength and pH constant. In addition, it was important to work at maximal extraction efficiency in the \((33\text{P})\)\textsubscript{P} extraction procedure. These goals were accomplished as follows. First, we performed the inhibi-
bition assays with only very limited ATP hydrolysis at each level of [ADP] and [ATP]. This led to a high extraction efficiency of hydrolyzed P, in the isobutanol/toluene extraction assay and circumvented any significant pH change. Second, ionic strength was kept constant by correcting the assay medium at each level of [ADP] and [ATP] with KCl or KP.

Another important feature in the design of the experiments was that, in order to maximize the interaction of actin and myosin, it was necessary to keep ionic strength low. This requirement severely limited the range of possible nucleotide and salt concentrations (including [KP] and [ADP]) that could be used. Therefore, instead of either monitoring complete hydrolysis of 1 mM ATP as in Figure 2 or keeping [ADP] constant while varying [ATP], we varied [ADP] and [ATP] from 0 to 1 mM such that the total nucleotide concentration, [ATP]+[ADP], was kept constant at 1 mM. The ionic strength was kept constant at 13 mM by using either KCl or KP, with the following assumptions: the ionic strengths of 1 mM MgATP and 1 mM MgADP are 5.0 and 4.0 mM, respectively, and the ionic strengths of 1 mM KP or KCl are 1.6 mM and 1 mM, respectively. In these experiments, less than 0.1 mM ATP was hydrolyzed at each particular [ADP] and [ATP], and the pH remained constant in the presence of 6–10 mM imidazole. In all experiments for which [ADP] and/or [ATP] were varied, the reported concentrations were corrected for ATP split during the experiment.

Figure 3 shows the normalized actin-activated ATPase for each protein, with the actin adjusted to a level that gave approximately half-maximal activation in the absence of added ADP (see Table 1). This provides a direct comparison of cardiac, smooth, and skeletal acto-S-1 at the $K_{ATPase}$ of each protein tested, i.e., at the same level of activation by actin. These data indicate that for a given level of actin activation, ADP inhibits the ATPase of cardiac and smooth muscle S-1 to a much greater extent than it does skeletal muscle S-1. Both cardiac and gizzard S-1 exhibited significant ATPase inhibition at low levels of [ADP] (≤ 0.5 mM), whereas skeletal S-1 did not. In addition, these data demonstrate that the inhibition observed in Figure 2 cannot be explained by changes in ionic strength or pH, since both were controlled in the experimental design.

**ATPase Inhibition Related to Substrate (ATP) Depletion**

We next attempted to determine how much of the observed inhibition could be due to substrate (ATP) depletion alone. In a separate set of paired experiments, [MgATP] was varied between 0.1 and 1.0 mM in the presence or absence of added MgADP, and ionic strength was maintained at a constant level using KCl. Actual [ATP] varied from 0.95 to 0.05 mM, once the correction was made for ATP split during the experiment. There was little or no decrease in ATPase activity in the absence of added ADP, even at [ATP] as low as 0.05 mM (Figure 4, upper curves). Thus, substrate depletion alone could not explain the reduced ATPase rates observed in the presence of increasing levels of [ADP] (Figure 4, lower curves).

**ATPase Inhibition Related to Increased [P]**

We then tested whether P could be responsible for part of the observed inhibitory effect on the ATPase activity. The strategy here was to detect an inhibitory response to [P] by working under conditions involving [ADP] in which significant inhibition was evident (i.e., at >0.5 mM ADP), and then vary [KP] by exchanging KP with KCl while keeping ionic strength constant. In this case, if P were responsible for at least part of the inhibition, a definite change in ATPase activity would be observed. Figure 5 shows that 1.5 mM KP had no significant effect compared with 2.4 mM KCl on the acto-S-1 ATPase activity for any of the three muscle types. Neither $V_{max}$ nor $K_{ATPase}$ was affected by KP. Thus, increasing [P] was not responsible for the inhibition observed in Figure 2. Other studies using cardiac S-1 and KP, concentrations up to 2.5 mM (requiring an
ADP Inhibition of the Actin-Activated ATPase Activity at Different Actin Concentrations

Figure 6 shows plots at “low,” “intermediate,” and “high” actin concentrations for the three S-1 types. The choices of actin concentrations were made to see if actin had any significant effect on the inhibition observed. The data were analyzed using a least-squares hyperbolic fitting program. This procedure determined the values for \( F_1 \) and \( F_2 \) from Equation 1 (see “Materials and Methods”). Table 2 lists these constants for the data in Figure 6.

It is evident from Figure 6 that the data fit a hyperbolic plot reasonably well, strongly supporting the prediction of the models used (Figure 1) that the relation between ATPase and [ATP] is hyperbolic. Table 2 also lists the ADP to ATP ratios that gave 50% inhibition of the rate observed at 1 mM ATP and 0 ADP ([ADP]/[ATP]_{50%}, see “Materials and Methods”). The analysis confirms our observation from Figures 3 and 6 that there is little or no ADP inhibition of skeletal S-1 ATPase activity, regardless of actin concentration. An ADP to ATP ratio of 40–50:1 would be necessary to obtain 50% inhibition of the skeletal S-1 ATPase activity. Cardiac and smooth muscle S-1 ATPase activities were inhibited 50% at quite low ADP to ATP ratios, 4–8:1 for cardiac muscle and 2–4:1 for smooth muscle. In addition, for cardiac and smooth muscle S-1, 50% inhibition occurred at smaller ADP to ATP ratios as the actin concentration was raised. This suggests that the inhibition affects mainly the acto-S-1 complex, and this finding fits well with the model proposed in Figure 1b.

We were also able to estimate values for \( K_a/K_i \) from the data in Figure 6 (see “Materials and Methods”). The value of \( K_a \) varies with the S-1 type as well as the actin concentration. To determine the ratio of these apparent activation constants, it is necessary to know \( K_a \). We do not have exact values for \( K_a \), which for this analysis is the [ATP] that gives one half of the product release rate at a given actin concentration. However, we can estimate \( K_a \) as follows. Acto-S-1 ATPase activity involves two series of steps: a rapid hydrolysis step (including ATP binding) and a product release step. Rapid hydrolysis is the step that is affected by [ATP], and this rate can be estimated by stopped-flow fluorescence burst measurements. By relating one half of \( V_{max} \) (from Table 1) to reported values for the \( K_a \) for the burst rate and fluorescence burst rates in the presence of actin (References 22 and 24 and L.A. Stein and J.S. Drew, unpublished data), we were able to estimate that \( K_a \) is approximately 1 \( \mu \)M for the three types of S-1 at infinite actin (0.5–2 \( \mu \)M for skeletal and cardiac S-1 and 0.25–1 \( \mu \)M for gizzard S-1). Therefore, if we ignore the small \( F_2/(K_a) \) term from Equation 4 (see “Materials and Methods”), the estimates for \( K_a/K_i \) will be within approximately 5%. This leaves us with estimates for \( K_a/K_i \) given by \( F_1/(F_1+F_2) \), which is equal to the inverse of [ADP]/[ATP]_{50%}, and these values are listed in Table 2. Furthermore, by using 1 \( \mu \)M as an estimate for \( K_a \), [ADP]/[ATP]_{50%} (Table 2) approximates \( K_a \) for ADP in the presence of actin.

Discussion

The present study demonstrates significant inhibition of the actin-activated MgATPase of cardiac and gizzard S-1 associated with an increase in the ADP to ATP ratio. Additional experiments demonstrated that neither high levels of [P] (1.5–2.5 mM) nor substrate depletion (low [ATP]) was associated with the observed ATPase inhibition for any S-1 type. As predicted by analysis of the kinetic model for ATPase activity (Figure 1b), there was a simple hyperbolic relation between [ATP] and ATPase activity, given that [ADP]+[ATP]=1 mM. Hyperbolic fits of the data indicated that 50% inhibition of the actin-activated ATPase activity occurred at an ADP-to-
FIGURE 4. Graphs showing effect of substrate (ATP) depletion on the actin-activated Mg\(^{2+}\) ATPase of skeletal (top panel), cardiac (middle panel), and gizzard (bottom panel) subfragment-1. ATPase activity was measured in the presence of decreasing amounts of ATP with or without increasing amounts of ADP added. Results are normalized to the initial rate in the presence of 1 mM ATP and 0 mM ADP. Conditions were as follows: 10 mM imidazole, 2 mM MgCl\(_2\), 1 mM dithiothreitol, and 1–0.1 mM ATP: [ATP] corrected for amount hydrolyzed by 5 minutes; and ionic strength maintained at 13 mM with added KCl. □, No ADP added; ■, 0–0.9 mM ADP added such that [ADP] + [ATP] = 1 mM.
ATP ratio of 3:1 for gizzard S-1 and an ADP-to-ATP ratio of 6:1 for cardiac S-1. By way of contrast, an ADP-to-ATP ratio of 40–50:1 would be required for 50% inhibition of skeletal muscle acto-S-1. Inhibition was greater at higher actin concentrations, suggesting that ADP is exerting its effect primarily on actin-bound complexes, but this is also partially due to the asymmetry in the model for ATP and ADP. ADP only associates and dissociates, whereas ATP is also hydrolyzed.

From Figure 1b (see also “Materials and Methods”), it is obvious that exact quantitation of $K_{\text{ADP}}/K_{\text{ATP}}$ is not possible until considerably more kinetic experiments are performed. Both the ATP and ADP “off” rates, $k_{\text{ATP}}$ and $k_{\text{ADP}}$, must be determined, and this is outside the scope of the present report. Preliminary studies in our laboratory confirm that the ADP “off” rate for porcine cardiac acto-S-1 at 15°C is approximately 60/sec, in close agreement with the value for bovine cardiac S-1 obtained previously. In addition, preliminary data with gizzard acto-S-1 at 25°C reveals an ADP “off” rate that is about two orders of magnitude faster than $V_{\text{max}}$ and, therefore, is not rate limiting for the ATPase cycle. Only one prior study to measure the ATP “off” rate exists in the literature, and it used quench-flow kinetics. The technique used in these experiments was a difficult one, and the interpretation of the data was model dependent and also depended on several assumptions. However, this technique can be adapted to our system, making it possible to develop an interpretation of the data according to the model in Figure 1b.

We were able to estimate $K_{\text{m}}/K_{\text{r}}$ to within approximately 5% for the three muscle types at different actin concentrations from the present data. This serves as a good approximation of the relative affinity of ADP versus ATP for the acto-S-1 active site. Qualitatively, these data indicate that the relative “affinity” for ADP versus ATP is quite strong for cardiac and smooth muscle acto-S-1 (approximately 0.2 and 0.3, respectively) but very weak (approximately 0.02) for skeletal acto-S-1. To estimate the value of $K_{\text{m}}$, we would require a determination of $K_{\text{r}}$. A good estimate of this constant would be difficult to obtain directly because at the very low [ATP] necessary to estimate $K_{\text{m}}$, the [ADP] resulting from hydrolysis would be high enough to inhibit the ATPase, especially for cardiac and smooth muscle S-1. However, we were able to estimate $K_{\text{m}}$ indirectly from other kinetic constants (see “Results”) and found that $K_{\text{m}}$ is approximately 1 μM for the three types of S-1 in the presence of actin. This gives us approximations for $K_{\text{r}}$ of 50, 4–8, and 2–4 μM for skeletal, cardiac, and gizzard S-1, respectively. These values compare favorably with $K_{\text{r}}$ values estimated from in situ studies with skeletal (120 μM)$^6$ and smooth muscle tissue (2–5 μM)$^1$ as well as an ADP association constant for cardiac acto-S-1 in vitro (4 μM).$^{13}$

Another point that needs to be examined is whether the new data presented here could have any effect on the prior interpretations of kinetic data with cardiac proteins from our laboratory. We do not believe this to be the case, because we were aware very early that the ATPase activity of cardiac acto-S-1 was initially linear and then appeared to slow down late in the reaction. Skeletal acto-S-1 also slowed down but not until 90+% of the ATP was split, whereas for cardiac S-1 it was clear that slowing occurred earlier. We also found very early that, unlike results obtained with skeletal proteins, the turbidity of cardiac acto-S-1 in the presence of ATP began to rise very slowly after approximately half of the ATP was split. Hence, all of our reported studies were carried out under conditions in which reasonable linearity was ensured, i.e., conditions in which less than 50% of the ATP was split and [ADP] was insufficient to lead to significant inhibition.

**Physiological Significance of ADP Inhibition**

Within the structural environment of the skeletal muscle fiber, high concentrations of ADP decrease ATPase activity but increase isometric tension. High concentrations of $P_i$, however, decrease tension and slightly decrease the isometric ATPase but do not inhibit $V_{\text{max}}$ in solution. These observations are consistent with a model suggesting that crossbridge attachment is weak before the release of phosphate and then strong while ADP remains bound. In this model, high concentrations of $P_i$ would favor the weakly bound A-M-ADP-P, state (low tension), whereas high [ADP] would favor the strongly bound A-M-ADP state (high tension, slow ATPase). In the solution system of biochemical kinetics, $[P_i]$ had little or no effect on ATPase activity, and this is probably due to the fact that the A-M-ADP state is always at its minimal free-energy conformation in solution as soon as $P_i$ is released. In the fiber, however, after $P_i$ is released, the A-M-ADP state is in a strong binding conformation far away from its minimal free-energy conformation, and work must be performed to bring the A-M-ADP state to its minimal free-energy state. For this reason, $P_i$ binding to A-M-ADP in solution can be very different from $P_i$ binding to the A-M-ADP state in vivo after $P_i$ is released.

Based on the existence of copious enzymes to convert ADP to ATP in skeletal muscle, it is generally accepted that [ADP] cannot increase sufficiently to cause ATPase inhibition in vivo. However, the situation for cardiac and smooth muscle may be quite different. In the living rat, hypoxic ventilation (8% or 10% $O_2$) results in severe depression of cardiac function, decline in high-energy phosphate (ATP and creatine phosphate) turnover, and an increase in [ADP] from 58% to 71% of total nucleotide. This value for [ADP] is in the range of significant ATPase inhibition in vitro (see Figure 6, middle panel). In addition, the measured nucleotide concentrations are tissue concentrations. Local [ADP] at the myofilament may be higher because of compartmentalization. Thus, ADP inhibition may contribute to the decline in high-energy phosphate turnover with cardiac hypoxia and could conceivably be involved in the impaired diastolic relaxation (prolonged strong binding state?) observed with myocardial ischemia.

In skinned smooth muscle tissue, elevated ADP causes contraction in the absence of calcium and myosin light chain (MLC) phosphorylation and is associated with increased $Ca^{2+}$ sensitivity and slow relaxation. In intact smooth muscle tissue, ATP and phosphocreatine tissue contents do not change significantly during development of isometric force, but there is a small but significant increase in ADP content. This again may reflect a larger increase at the contractile apparatus that is due to functional compartmentalization, with the possibility that ADP reaches concentrations high enough (≥50% of total nucleotide, according to our in
FIGURE 5. Graphs showing effect of inorganic phosphate (Pi) on the actin-activated Mg$^{2+}$ATPase of subfragment-1. Steady-state ATPase measurements were obtained in the presence of ADP with added KP$_i$, KCl, or imidazole. Lines are double reciprocal plots of hyperbolic fits of the raw data. Conditions were as follows: 2 mM MgCl$_2$ (1=m-free), [ADP]+[ATP]=1 mM, 1 mM dithiothreitol, approximately 15 mM ionic strength, and KP$_i$, KCl, and imidazole as indicated. Top panel: Skeletal subfragment-1 with 8 mM imidazole, 0.2 mM ATP, and 0.8 mM ADP, along with 1.5 mM KP$_i$ (●) or 2.4 mM KCl (×) at 15°C. Middle panel: Cardiac subfragment-1 with 0.25 mM ATP and 0.75 mM ADP, along with 10 mM imidazole and 0.75 mM KP$_i$ (●), 7 mM imidazole and 1.75 mM KP$_i$ (●), or 10 mM imidazole and 1.125 mM KCl (×) at 15°C. Bottom panel: Gizzard subfragment-1 with 0.4 mM ATP, 0.6 mM ADP, and 10 mM imidazole, along with 1.5 mM KP$_i$ (●) or 2.4 mM KCl (×) at 25°C.
FIGURE 6. Graphs showing inhibition of the actin-activated Mg\textsuperscript{2+}ATPase of subfragment-1 with increasing concentrations of ADP and varied concentrations of actin. ATP hydrolysis was monitored with varying concentrations of ADP and ATP. The total nucleotide concentration was kept constant at 1 mM (i.e., [ADP]+[ATP]=1 mM), and only ≤10% of the ATP was hydrolyzed during the time course of the experiments. Lines are hyperbolic least-squares fits of the data. Conditions were as follows: 10 mM imidazole, 2 mM MgCl\textsubscript{2} (1=free), 1 mM dithiothreitol, and [ADP]+[ATP]=1 mM. Top panel: Skeletal subfragment-1 (0.1 μM) and 12 (×---×), 5 (■---■), or 2 (+---+) μM actin at 15°C. Middle panel: Cardiac subfragment-1 (0.1 μM) and 24 (■---■), 8 (×---×), 4 (+---+), or 2 (■---■) μM actin at 15°C. Bottom panel: Gizzard subfragment-1 (0.2 μM) and 20 (■---■), 10 (×---×), or 5 (+---) μM actin at 25°C.
Table 2. Values Derived From Hyperbolic Fits of Data From Figure 6

<table>
<thead>
<tr>
<th>Muscle type</th>
<th>[Actin] (μM)</th>
<th>F₁</th>
<th>F₂</th>
<th>[ADP]/[ATP] slopes*</th>
<th>Kₐ/K₁†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal</td>
<td>2</td>
<td>0.875</td>
<td>0.018</td>
<td>49:1</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.509</td>
<td>0.014</td>
<td>38:1</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.411</td>
<td>0.009</td>
<td>47:1</td>
<td>0.021</td>
</tr>
<tr>
<td>Cardiac</td>
<td>2</td>
<td>1.616</td>
<td>0.226</td>
<td>8:1</td>
<td>0.123</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.027</td>
<td>0.198</td>
<td>6:1</td>
<td>0.162</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.774</td>
<td>0.146</td>
<td>6:1</td>
<td>0.159</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.544</td>
<td>0.174</td>
<td>4:1</td>
<td>0.242</td>
</tr>
<tr>
<td>Smooth</td>
<td>5</td>
<td>1.548</td>
<td>0.497</td>
<td>4:1</td>
<td>0.243</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.080</td>
<td>0.476</td>
<td>3:1</td>
<td>0.306</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.435</td>
<td>0.473</td>
<td>2:1</td>
<td>0.521</td>
</tr>
</tbody>
</table>

F₁ and F₂, defined by \( V_{\text{ATP}} = F_1 + (F_2 / [ATP]) \) where \( V_{\text{ATP}} \) is the observed ATPase rate; [ADP]/[ATP] slopes* ratio that gives 50% inhibition of the ATPase rate observed at 1 mM ATP, given [ADP]/[ATP]=1 mM; \( K_m \), apparent activation constant of the actin-activated ATPase activity for ATP; \( K_a \), apparent inhibition constant of the actin-activated ATPase activity for ADP. Values were derived given that \( V = F_1 + (F_2 / [ATP]) \) where \( V \) is the observed ATPase rate.

*Value estimated by \( (F_1 + F_2)/F_2 \). See text for derivation. Assuming \( K_m \) is approximately 1 μM, this value also provides an estimate (in μM) for \( K_a \).
†Value estimated by \( F_2/(F_1 + F_2) \). See text for derivation.

vitro data) to modulate force, ATPase activity, and shortening velocity in vivo.

Some smooth muscles, including gizzard, have a biphasic pattern of contraction. An initial transient phase characterized by high [Ca^{2+}], high levels of MLC phosphorylation, and rapid shortening velocity and stress development is followed by long-term stress maintenance at low (but suprabasal) levels of [Ca^{2+}], MLC phosphorylation, ATPase activity, and shortening velocity. The second phase, termed "latch," has been adequately described by a four-state physiological model in which phosphorylated crossbridges maintain force with rapid crossbridge cycling while dephosphorylated crossbridges maintain force with very slow crossbridge detachment. Although the latch state has been described well physiologically, the biochemical basis for this phenomenon remains obscure. For example, it is not understood how unphosphorylated crossbridges would maintain tension at low but suprabasal levels of [Ca^{2+}] but permit fast relaxation at basal [Ca^{2+}]. Proposed calcium-regulated thin-filament proteins such as caldesmon or calponin may be involved. Additionally or alternatively, an increase in the ADP to ATP ratio occurring with activation may promote or prolong tension maintenance by unphosphorylated crossbridges.

The latch model predicts a linear relation between shortening velocity and MLC phosphorylation. However, when comparing the relation between shortening velocity and MLC phosphorylation for various tissue types, there are variations that cannot be explained by the small observed differences in the inherent ATPase rates of the purified myosins. In addition, there are differences in the relation between shortening velocity and MLC phosphorylation in a given tissue when stimulated by different agonists. Again, these differences may be related to Ca^{2+}-regulated thin-filament proteins, but ADP may be an additional or alternative modulator of the crossbridge cycling rate.

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References


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