Effect of Ouabain on the ATPase of Cardiac Myosin B at High Ionic Strength

By Ada L. Jacobson, Ph.D.

ABSTRACT
The effect of ouabain (10^{-8} to 10^{-6} M) on the hydrolysis of ATP by beef cardiac myosin B was measured at various KCl concentrations and at various interaction times at 25°C. Using an ionic strength of 0.24 M KCl and a 10-minute contact time, 10^{-7} and 10^{-5} M ouabain approximately doubled the ATPase activity of freshly prepared natural actomyosin. After storage of the myosin B sample in 0.6 M KCl at 2°C, ouabain had a small inhibitory effect in 0.24 M KCl on the ATPase activity. At an ionic strength of 0.36 M KCl, only the inhibitory effect was observed. This effect was dependent on contact time and on calcium ion concentration. Previous treatment of the myosin B with EDTA removed the activating effect observed in 0.24 M KCl at short contact times. Addition of 0.1 mM MgCl_{2} restored the activating effect of ouabain. In the EDTA-treated myosin B in 0.6 M KCl at high ouabain concentrations in the presence of 1 mM CaCl_{2}, a small activation was observed. Addition of 0.1 mM MgCl_{2} under the same experimental conditions produced a small deactivation. The effect of ouabain on the ATPase activity of myosin B in solution appears to be dependent on protein preparation, age of the protein preparation (which may affect aggregation), ionic strength, ouabain concentration, contact time, and divalent ions present. All effects induced by ouabain on the ATPase activity of myosin B may be related to the conformation of the myosin B in solution. However, no simple relationship was observed with any individual variable such as preparation, age, contact time, or ouabain concentration.

ADDITIONAL KEY WORDS actomyosin myosin-actin junctions KCl cardiac glycoside-protein Ca^{2+} concentration

Various possible mechanisms and interaction sites by which cardiac glycosides could affect the contractile process have been postulated (1). The inotropic action of cardiac glycosides may be due to changes in the ion transport system in muscle (2-4) or to direct interaction of cardiac glycosides with the contractile proteins. Robb and Mollov (5) demonstrated an increased shortening when ATP was added to artificial actomyosin threads prepared from solutions containing ouabain. In a personal communication Robb has indicated that this increased shortening was not observed in threads formed from pure actomyosin solutions when ouabain and ATP were added together after formation of the fibers.

These observations indicate that incorporation of the cardiac glycoside into the thread was necessary for increased shortening, and surface contact was only a minor factor. Waser (6) has measured the direct binding of cardiac glycosides to actomyosin.

Conflicting results have been reported on the effect of ouabain on the enzymatic activity of actomyosin. It is possible that these discrepancies may be due to differences in the experimental conditions or to differences in actomyosin due to variations in the method of preparation. Stowring and associates (7) reported that ouabain increased both super-precipitation and ATPase activity of myosin B in 0.12 M KCl. While relatively large variations occurred from sample to sample, the general effect averaged over a large number of samples was unquestionable. However Kay and Green (8) had previously reported that in myosin B solutions with 0.5 M KCl ouabain
had no effect on the ATPase activity or other physical properties. Katz (9) has also reported that ouabain produced no effect on either the ATPase activity or the superprecipitation of synthetically prepared actomyosin in 0.08 M KCl.

In this investigation the ATPase activity of myosin B has been studied in the presence of ouabain under various experimental conditions to isolate the variables which may influence the effect of ouabain and to rationalize the conflicting results reported by other investigators. The physical state of the myosin B can be influenced by a number of factors such as the method of extraction, the time of extraction (10), and the order of dilution and centrifugation (11). In addition, aggregation occurs in solution (12) which may or may not be reversible, depending on experimental conditions (13). At low ionic strength, when myosin B is in a precipitated or gel form, physical differences among samples due to particle size are inevitable. Noda and Maruyama (14) have shown that at concentrations greater than 0.3 M KCl, the myosin B is in solution but at 0.2 to 0.3 M KCl it may be in a gel or precipitated state, depending on the experimental conditions.

To eliminate the possibility of physical differences due to particle size, in this investigation the ATPase activity of myosin B was measured only with myosin B in solution. The lowest ionic strength used was 0.24 M KCl; no gel or precipitate was visible in any of these experiments. Myosin B solutions were also studied at 0.36 and 0.60 M KCl. The preparation technique, the storage time of the myosin B, the contact time between the ouabain and the myosin B, and the divalent ion concentrations have all been varied in order to determine the effect of the physical state of the myosin B on any changes in ATPase activity caused by ouabain.

Materials and Methods

Beef cardiac myosin B was prepared by the method described by Stowring and associates (7). The extraction time for these preparations was 22 hours. This method of preparation was modified only for the experiments reported in Figures 4 and 5. These experiments were designed to explore the effect of preparation and of divalent ions on the influence of ouabain on the ATPase activity of myosin B, and 0.01 M EDTA was added to the extracting solution used by Stowring and associates to remove divalent cations. The same purification techniques were then used. However, to remove any trace of the EDTA, the final protein solution was dialyzed against 0.6 M KCl.

Stock solutions of proteins were stored at pH 7 in 0.6 M KCl at 2°C. Protein concentrations were determined by micro-Kjeldahl measurements. The factor 0.25 was used to convert nitrogen concentration to protein concentration. The stock solution was diluted with buffer solution immediately prior to the ATPase measurements. No precipitation was seen in any of the diluted solutions. Solutions were stirred to obtain temperature equilibrium but not treated in any other manner. Analytical reagent grade salts and glass double-distilled water were used throughout. Crystalline ouabain was obtained from the Nutritional Biochemical Co. Disodium ATP was obtained from Sigma Chemical Co.

The ATPase activity was determined at 25°C by measuring the rate of liberation of inorganic phosphate according to the method of Fiske and SubbaRow (15). To determine the effect of contact time with ouabain, the diluted myosin B was warmed to 25°C and ouabain added simultaneously with the ATP for the short contact-time experiments. In other experiments, the diluted myosin B was warmed to 25°C, ouabain was then added, and after a fixed length of time the ATP was added. Aliquots were taken in all ATPase experiments at 2-minute intervals from 4 to 14 minutes after additions of ATP.

All measurements reported are an average of data from at least two experiments. Most experiments concerning the effect of ouabain on the ATPase activity of myosin B were repeated four or five times. The standard deviations reported have all been calculated from the formula

$$\sqrt{\frac{\sum (X-\bar{X})^2}{N-1}},$$

which is appropriate for a small number of measurements. All samples contained 0.02 M Tris and 2 mM ATP. The protein concentration was varied by dilution to determine the linear range of activity with 2 mM ATP. Within this range, the ATP concentration was varied to check further whether maximal ATPase activity was obtained. The reference samples of the myosin B (no ouabain) were always at the same pH and at the same ionic strength with the same concentration of divalent cations and were treated...
TABLE 1

Activity as a Function of Dilution of the Myosin B Stored for 3 Days at 2°C in 0.6 M KCl

<table>
<thead>
<tr>
<th>Protein concentration mg/ml</th>
<th>μM Pi/g/sec mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.250</td>
<td>2.58 ± .08</td>
</tr>
<tr>
<td>0.125</td>
<td>2.66 ± .08</td>
</tr>
<tr>
<td>0.100</td>
<td>3.14 ± .08</td>
</tr>
<tr>
<td>0.050</td>
<td>4.08 ± .21</td>
</tr>
<tr>
<td>0.0250</td>
<td>4.04 ± .13</td>
</tr>
<tr>
<td>0.0125</td>
<td>3.93 ± .10</td>
</tr>
</tbody>
</table>

Myosin B preparation: 0.24 M KCl, 10 mM CaCl₂, 0.02 M Tris, 2 mM ATP, at 25°C.

In exactly the same manner as, and always measured simultaneously in the same experimental sequence with, the samples containing ouabain.

Results

The linear range of protein concentration for measurement of the ATPase activity of the myosin B was determined by dilution for each protein preparation. A sample determination is shown in Table 1. The ATP concentration was then varied to ensure that maximal ATPase activity had been obtained. For this particular protein preparation, an increase from 1 to 3 mM ATP at a protein concentration of 0.05 mg/ml produced no change in the rate (4.0, 4.1, 4.0 μM Pi/g per sec ± 0.2 (sd) for 1, 2, and 3 mM ATP, respectively).

The effect of protein storage on the ATPase activity in a typical protein sample is shown in Table 2. The enzymatic activity of the myosin B decreased on storage at 2°C in 0.6 M KCl at pH 7.0 at a concentration of 2 to 3 mg/ml. Immediately before measurements of ATPase activity, the samples were diluted to 0.24 M KCl. The number of determinations, the mean value of the ATPase activity, and the standard deviations for each set of measurements are given in Table 2. An initial large decrease in activity was followed by a slower decrease with further storage. The effect of such storage on the ATPase activity in the presence of 10⁻⁸ M ouabain is also shown in Table 2. The myosin B-ouabain contact time was 10 minutes at 25°C. With the fresh myosin B preparation, the rate of hydrolysis of ATP was approximately half in the presence of 10⁻⁸ M ouabain. The change in activity caused by ouabain was significantly greater than the experimental error as indicated by the standard deviation. After 4 days of storage the effect of ouabain decreased compared to the reference samples at the same age, and nearly 80% of the activity remained. With longer storage, the ouabain addition increased the ATPase activity of the myosin B. In general, the aging effects on the enzymatic activity of the myosin B were less marked in the presence of 10⁻⁸ M ouabain. However, as shown below, this effect may be due to the particular ouabain concentration employed in the experiments, as the effect of ouabain on the ATPase activity of the myosin B also depended on the ouabain concentration.

With freshly prepared myosin B (less than 24 hours of storage at 2°C in 0.6 M KCl) the rate of hydrolysis of the ATP in 0.24 M KCl

TABLE 2

Activity as a Function of Storage of the Myosin B Preparation at 2°C in 0.6 M KCl

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>N₁</th>
<th>μM Pi/g/sec mean ± SD</th>
<th>N₂</th>
<th>10-min stirring with 10⁻⁸ M ouabain μM Pi/g/sec mean ± SD</th>
<th>Relative activity 10⁻⁸ M ouabain addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>6.37 ± .42</td>
<td>4</td>
<td>3.44 ± .43</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>4.08 ± .21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>3.43 ± .13</td>
<td>2</td>
<td>2.65 ± .08</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>2.91 ± .17</td>
<td>5</td>
<td>3.24 ± .21</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>2.34 ± .15</td>
<td>5</td>
<td>3.16 ± .12</td>
<td>1.3</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>2.06 ± .10</td>
<td>3</td>
<td>3.05 ± .08</td>
<td>1.5</td>
</tr>
</tbody>
</table>

N₁ = number of observations in the absence of ouabain. N₂ = number of observations in the presence of ouabain. Myosin B preparation: 0.24 M KCl, 10 mM CaCl₂, 0.02 M Tris, 0.05 mg myosin B/ml, pH 7.4, 2.0 mM ATP, T = 25°C for ATPase determinations.

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Effect of ouabain on the ATPase activity of beef cardiac myosin B in 0.24 M KCl, 0.02 M Tris, 2 mM ATP, pH 7.4, 0.005% protein, 10 mM CaCl₂, 10-minute ouabain-myosin B contact time, 25°C. 0 = freshly prepared myosin B; □ = myosin B stored 4 days at 2°C in 0.6 M KCl at pH 7.0 before use.

Depended markedly on the ouabain concentration. In Figure 1 the rate of hydrolysis of ATP by freshly prepared and aged myosin B in 0.24 M KCl is shown as a function of ouabain concentration. The mean standard deviation calculated for each concentration is less or equal to ±0.2 μM P_i/g/sec in all cases. With the freshly prepared myosin B there were two peaks of activity at 10⁻¹ and 10⁻² M ouabain. A definite lowering of the rate of hydrolysis of ATP by myosin B was observed at 10⁻⁸ and 10⁻⁹ M ouabain. However, relatively little change was observed as a function of ouabain concentration for stored myosin B.

To determine whether the ouabain-myosin B contact time was a factor in determining the effect of ouabain on the ATPase activity of myosin B, the contact time was varied from a short time to a 10-minute stirring with ouabain before ATP was added. The results shown in Table 3 are for myosin B stored at 2°C for 6 days. While there appears to be no regular progression in the effect of contact time with ouabain on the ATPase activity of myosin B, it appears clear that the rate of hydrolysis of the ATP depends on the length of time the myosin B and the ouabain are incubated together.

To determine whether ionic strength was also an important variable in the effect of ouabain on the ATPase activity of myosin B, the ionic strength was increased to 0.36 M KCl. At this higher ionic strength, with both a low contact time and a 10-minute contact time, the ouabain decreased the rate of hydrolysis of ATP; with the low contact time this decrease was small while with the 10-minute contact time it was large (Fig. 2). The

### Table 3

<table>
<thead>
<tr>
<th>Contact time (min)</th>
<th>No. of observations</th>
<th>μM P_i/g/sec mean value ± SD</th>
<th>Relative activity due to ouabain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>2.14 ± 0.17</td>
<td>1.1</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>2.45 ± 0.17</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>3.19 ± 0.23</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>3.09 ± 0.11</td>
<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>2.55 ± 0.13</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>2.07 ± 0.20</td>
<td>0.9</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>2.90 ± 0.13</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*Before addition of ouabain.
†Using the t-test a highly significant difference at the 99% level was found between the means of the samples with ouabain and without ouabain.

Myosin B preparation: 0.24 M KCl, 10 mM CaCl₂, 0.02 M Tris, 0.05 mg myosin B/ml, pH 7.4, 2.0 mM ATP, at 25°C for ATPase determination.
Relative change in ATPase activity of beef cardiac myosin B on addition of ouabain in 0.36 M KCl, pH 7.4, 0.02 M Tris, pH 7.4, 0.013% freshly prepared protein, 10 mM CaCl₂, 25°C. Initial activity of preparation 1 was 5.8 μM P₄/g/sec and for preparation 2, 5.4 μM P₄/g/sec.

- • = low ouabain-myosin B contact time (simultaneous addition), average value of relative change for two preparations (deviation in relative change in ATPase activity for low contact time is ±0.03); • = 10-minute contact time for preparation 1; □ = 10-minute contact time for preparation 2.

**Figure 2**

Effect of ouabain on the ATPase activity of beef cardiac myosin B treated with EDTA in the extraction process. Solutions were extensively dialyzed before use; 0.24 M KCl, 0.02 M Tris, pH 7.4, 0.014% protein, short ouabain-myosin B contact time, 25°C. ○ = 1 mM CaCl₂ and no MgCl₂; □ = 1 mM CaCl₂ and 0.1 mM MgCl₂; broken line = 1 mM CaCl₂ and 0.1 mM MgCl₂, protein stored (2°C, 0.6 KCl, pH 7) for 4 days previous to measurement.

**Figure 4**

Relative change in ATPase activity of cardiac myosin B on addition of ouabain in 0.36 M KCl, 0.02 M Tris, pH 7.4, 0.013% freshly prepared protein, 10-minute ouabain-myosin B contact time, 25°C. ○ = 10 mM CaCl₂ (initial activity 5.8 μM P₄/g/sec); □ = 1 mM CaCl₂ (initial activity 4.8 μM P₄/g/sec).

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trend is similar with both preparations. The trend is clearly different from the results in 0.24 M KCl for fresh myosin B under otherwise identical conditions (circles, Fig. 1).

The concentration of calcium chloride present appears to be an additional factor which influences the effect of ouabain on the ATPase activity of myosin B in 0.36 M KCl. The relative changes in the ATPase activity of freshly prepared myosin B caused by 10 minutes of stirring with ouabain are shown in Figure 3. With both 1 and 10 mM CaCl₂, the ouabain generally decreased the hydrolysis of ATP by myosin B. The change in relative activity does appear to depend on the particular calcium chloride concentration.

The conflicting results reported in the literature may be due to differences in the protein due to preparation or to differing concentrations of intrinsically bound ions. Addition of EDTA to the extracting solution produced a protein preparation with different enzymatic
Effect of ouabain on the ATPase activity of freshly prepared beef cardiac myosin B treated with EDTA in the extraction process. Solutions were extensively dialyzed before use; 0.6 M KCl, 0.02 M Tris, pH 7.4, 0.01% protein, short ouabain-myosin B contact time, 25°C. o = 1 mM CaCl$_2$ and no MgCl$_2$; a = 1 mM CaCl$_2$ and 0.1 mM MgCl$_2$.

Properties in the presence of ouabain compared to protein prepared by the technique of Stowring and associates (7). As indicated in Figure 4, in freshly prepared myosin B which had been treated with EDTA to remove residual cations in 0.24 M KCl and 1 mM CaCl$_2$, no large change in activity was observed when ouabain was added with the ATP. The results resemble the changes observed in aged myosin B (squares, Fig. 1). This is in contrast with the large increase in rate of hydrolysis of ATP indicated in Figure 1 with the fresh actomyosin prepared by the method of Stowring and associates (7). However, addition of 0.1 mM MgCl$_2$ to the EDTA-treated myosin B restored the activation effect of ouabain.

To further illustrate the effect of ionic strength and of divalent cations, the ionic strength of the myosin B which had been extracted in the presence of EDTA was increased to 0.6 M KCl. As shown in Figure 5, with 1 mM CaCl$_2$, ouabain induced a small activation only at its higher concentrations. Addition of 0.1 mM MgCl$_2$ to these solutions reversed the behavior, and a deactivating trend was observed at the higher concentrations. The pattern of behavior as a function of ouabain concentration was completely different in 0.6 M KCl than in 0.24 M KCl.

**Discussion**

At the lowest ionic strength studied in this report (0.24 M KCl), there were two distinct concentrations of fresh myosin B at which ouabain essentially doubled the rate of hydrolysis of ATP. At a lower ionic strength of 0.12 M KCl with a contact time of 90 to 120 seconds, Stowring and associates (7) also observed a large increase in the rate of hydrolysis of ATP. In the previous report, the optimal ouabain concentration was 10$^{-6}$ and 10$^{-3}$ M; with our samples of myosin B the optimal ouabain concentrations were usually 10$^{-7}$ and 10$^{-5}$ M. Stowring and associates (7) also reported that the optimal concentrations did vary with protein preparations. The differences in optimal ouabain concentration could be ascribed to either differences in ionic strength or sample variation. The myosin B used by Stowring and associates (7) was treated by cavitation induced by ultrasound to ensure a uniform dispersion. In 0.24 M KCl in our preparations, no precipitation was observed and the solutions were stirred without any further treatment. It is possible that the ultrasonic treatment may affect the aggregation and might alter the ATPase activity observed, especially with the aged myosin B.

The effect of ouabain on the rate of ATP hydrolysis by freshly prepared myosin B in 0.36 M KCl is drastically different than in 0.12 and 0.24 M KCl. Ouabain inhibited the ATPase activity, and the effect was dependent on the calcium ion concentration (as well as the contact time). In 0.36 M KCl the myosin B would be expected to be in "solution" or in a form more like "solution" than it is in 0.24 M KCl. The difference in behavior may be due to a change in conformation due to change in ionic strength, which could be affected by the divalent ions present in solution.

With both 0.24 and 0.36 M KCl solutions, the contact time of the myosin B with the ouabain affected the rate of hydrolysis of...
ATP. This suggests that either the rate of binding of ouabain to the myosin B may be slow and ATPase site may be affected by the binding, or that a slow conformation change or a change in aggregation may occur in the myosin B which is ouabain sensitive or ouabain induced and this in turn may affect the ATPase site. The myosin B solutions used in the ATPase measurement were prepared by diluting a stock myosin B solution. This dilution would affect the aggregation. It is possible that the contact-time dependence is due to an effect of ouabain on the rate of change of aggregation during the approach to the equilibrium aggregation or to a change in the equilibrium aggregation itself after dilution. The contact-time change appeared to be dependent only on presence of ouabain.

Stowring and associates (7) reported that with myosin B in 0.12 M KCl the calcium, magnesium, and EDTA effects were additive on the effect produced by ouabain on the rate of both superprecipitation and ATPase activity of myosin B. These observations would appear to apply only to actomyosin at relatively low ionic strength, since at the higher ionic strength used here, treatment of the myosin B with EDTA in 0.24 M KCl completely removed the ouabain-induced activation in the presence of 1 mM CaCl₂. However, the addition of 0.1 mM MgCl₂ restored the activation effect. This suggests that the magnesium ion may be necessary for the myosin B-ouabain ATPase effects, and supports the hypothesis that the effect occurs at the myosin-actin junctions which are activated by magnesium ions. The absence of any effect of ouabain on the ATPase activity of myosin B in 0.08 M KCl reported by Katz (9) may be due, as suggested by Stowring and associates (7), to differences in the myosin-actin junction between synthetic and natural actomyosin, but also may be due to contact time or aging effects.

When the myosin B was in a true solution form in 0.6 M KCl, in the EDTA-treated myosin B, 0.1 mM MgCl₂ reversed the trend observed with 1.0 mM CaCl₂, also indicating that magnesium is directly involved at the ouabain-myosin B interaction sites. The difference in behavior when the KCl concentration is increased from 0.24 to 0.6 M also indicates that the conformation or aggregation of the myosin B may be a controlling factor on the effect of ouabain on the rate of phosphate liberation from ATP.

The change in the effect of ouabain on the hydrolysis of ATP by myosin B with age of the myosin B preparation is further supporting evidence. The aggregation with age of myosin B stored in 0.6 M KCl has been previously observed (13). The relatively small changes in the ATPase activity of the aged myosin B in the presence of ouabain reported in Table 2 may be due to the influence of ouabain on aggregation. However, it is possible that this smaller change in activity with age was observed because of the contact time chosen for the experiments (10 minutes), and much larger differences may be observed with different contact times.

The experiments reported in our work indicate that the determination of the effect of ouabain in the ATPase activity of myosin B is a very complex problem. Protein preparation technique, aging effects on the myosin B during storage, ionic strength, divalent ion concentration, ouabain-myosin B contact time and ouabain concentration, all affect the influence of ouabain on the ATPase activity of myosin B. Variation in protein samples due to preparation, other experimental conditions, or both, can vary the ouabain effect on the ATPase activity of myosin B from no measurable effect to either activation or inhibition.

It is possible that the negative results reported by Kay and Green (8) and also by Katz (9) and the positive results of Stowring and associates (7) and the author can all be rationalized on the basis that: (1) The conformation of myosin B controls any effect induced by ouabain on the hydrolysis of inorganic phosphate from ATP by myosin B. The conformation or the state of aggregation of the myosin B is a function of ionic strength, temperature, and age of the preparation. (2) For myosin B in solution, the divalent salt.
present may affect the conformation or aggregation. In the precipitated state, the effect of such ions may be of less importance. (3) The effect induced by ouabain on the ATPase of myosin B depends on the contact time between the myosin B and the ouabain. This may be due to slow binding of the ouabain or to a change in the conformation or state of aggregation of the myosin B in the presence of ouabain.

References
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