Action of Caffeine on Calcium Transport by Isolated Fractions of Myofibrils, Mitochondria, and Sarcoplasmic Reticulum from Rabbit Heart

LYNDA BLAYNEY, HUW THOMAS, JOHN MUIR, AND ANDREW HENDERSON

SUMMARY We studied the effects of caffeine on calcium transport by subcellular organelles isolated from rabbit myocardium. Caffeine increased myofibrillar basic and calcium-activated ATPase activity at 20 mM but not at lower concentrations. Mitochondrial and sarcoplasmic reticulum (SR) calcium accumulation was measured both by dual wavelength spectrophotometry with the calcium-sensitive dye, murexide, and by Millipore filtration with $45_{Ca}$. In mitochondria, caffeine impaired phosphate-assisted calcium transport but did not alter the closely related parameters of oxygen uptake, P/O ratio (nmol adenosine diphosphate consumed/n ats oxygen consumed, state 3 respiration) or limited calcium loading. In SR, caffeine impaired calcium accumulation. New methods were used to characterize calcium accumulation in the absence of oxalate according to first order reaction kinetics. Caffeine increased the rate constant while decreasing the calcium accumulated. It also increased the associated calcium-activated ATPase activity at low (30 μM) but not high (240 μM) external calcium concentration.

CAFFEINE is useful to demonstrate the role of the sarcoplasmic reticulum (SR) in excitation-contraction coupling in skeletal muscle. The effects of caffeine on the twitch contraction of mammalian heart muscle are more complex, being frequency and species-dependent. This has been explained on the basis of a dual action whereby caffeine augments calcium influx during the action potential and reduces the contribution of cellular calcium stores to activation. A general characteristic of caffeine’s action is that it slows mechanical relaxation, though notably not in amphibian heart muscle in which the SR is relatively less important. The circumstantial evidence thus supports the idea that caffeine impairs calcium sequestration by cardiac SR and that it interferes in some related way with the replenishment of available calcium stores.

The effects of caffeine on isolated SR preparations from skeletal muscle have been studied widely. Its effects on ATPase activity, ATP/Ca transport ratio, and calcium accumulation, with and without oxalate, support the view that caffeine acts by making the SR membrane more permeable to calcium. Its influence on the rate of the calcium pump and the passive flux of calcium is modulated by the relative internal and external calcium ion concentrations.

In isolated SR preparations from cardiac muscle, the mode of action of caffeine on calcium accumulation remains uncertain, as indeed does the physiological meaning of the in vitro process of calcium "binding” and calcium “uptake”. It has been suggested that caffeine may act in a qualitatively different way on SR isolated from different mammalian species, because of observations that it decreased calcium "binding” by the SR from rats but not from guinea pig hearts, whereas it decreased calcium "uptake” with oxalate in both species. Uncertainties remain also about the relative importance of SR and mitochondria in beat-to-beat regulation of the myoplasmic calcium concentration responsible for contraction and relaxation, and there is some conflicting evidence that caffeine may influence the transport of calcium by mitochondria.

In this study we have further characterized the effects of caffeine on intracellular calcium transport in heart muscle by comparing its effects on isolated SR and mitochondrial preparations. In particular, we have analyzed the kinetics of calcium accumulation of SR, using a modification of the murexide method. The findings suggest that caffeine acts in cardiac as in skeletal muscle by making the SR membrane more permeable to calcium.

Methods

Preparation of Subcellular Fractions

New Zealand white rabbits (2–3 kg) were killed by stunning. The heart was removed quickly and
placed in the appropriate ice-cold isolation buffer for the subcellular fraction being prepared. Atria and connective tissue were removed and only the ventricles were used for the experiments. Standard methods were used for the preparation of fractions rich in myofibrils, mitochondria, and microsomes. Myofibrils were prepared by repeated centrifugation with sucrose to reduce mitochondrial contamination, as previously described. Azide inhibition of calcium-activated ATPase activity in this fraction was 34.0 ± 2.1% (n = 6) (representing mitochondrial contamination), and 5'-nucleotidase activity was only 4.0 ± 0.18 nmol/mg protein per min (representing presumed sarcolemmal contamination, although no purified sarcolemmal preparation was available for comparison). Mitochondria were prepared by the method of Lindenmayer et al. using bovine serum albumin in the buffer to prevent damage by free fatty acids released. The mitochondrial fraction contained 13.9 ± 0.4 nmol/mg protein per min of 5'-nucleotidase activity (no satisfactory enzyme markers for myofibrillar or SR contamination are available). The microsomal fraction rich in SR was prepared by the method of Harigaya and Schwartz. Mitochondrial contamination in this fraction was assessed as 3% from measurements of succinic cytochrome c reductase activities in our mitochondrial and microsomal preparations (113.2 ± 8.8 and 3.5 ± 0.1 μmol/mg protein per min, respectively, n = 6); the presence of some sarcolemmal contamination was reflected by 75.4 ± 4.4 nmol/mg protein per min (n = 6) of 5'-nucleotidase activity, although no ouabain-sensitive Na+ K+ ATPase activity was detected.

**ATPase Assays**

Assay methods for ATPase activity in myofibril, mitochondrial, and SR preparations are given in Table 1. The reaction mixture was always preincubated for 5 minutes at the temperature to be used. The reaction was started by the addition of ATP and stopped by the addition of ice-cold 25% trichloroacetic acid (wt/vol) to a final concentration of 5% (vol/vol). The precipitate was removed by centrifugation and the phosphate content of the supernatant extract was assayed colorimetrically. The standard curve for phosphate concentration remained unaltered by caffeine (10 mM) or murexide (0.2 or 0.4 mM). For the caffeine studies, caffeine was added to the reaction mixture at the beginning of the 5-minute preincubation.

**Mitochondrial Respiration**

Oxygen uptake (state 3 respiration rate) and P/O ratio, using glutamate as the substrate, were measured at 30°C by the method of Lindenmayer et al. The reaction was started by adding ADP. The respiratory control index was the ratio of the state 3/state 4 respiration rates. For the caffeine studies, caffeine was added to the reaction mixture at the beginning of a 5-minute preincubation period.

**Calcium Transport-Murexide Method (Mitochondria and SR)**

A dual wavelength spectrophotometer (purpose-built) was used to study calcium ion transport, with an Amino-Morrow stopped-flow attachment for rapid mixing (< 4 msec). The stopped-flow attachment had two reservoir syringes: one (A) contained the organelles (± caffeine) in isotonic buffer (c. 1 ml); the other (B) contained all the other reactants in isotonic buffer (c. 1 ml). The final concentrations in the reaction chamber (c. 0.1-0.3 ml) after mixing are given in Table 2. The light path was 1 cm long. The difference in absorbances at 474 and 542 nm was used to monitor the concentration of Ca2+ with murexide. The signal was calibrated separately in each different reaction mixture for the free calcium concentration, using unbuffered calcium solutions of 15, 30, 60, and 120 μM (the limit of resolution was 5 μM). The signal was recorded with a Bryant's

**Table 1 Reaction Mixtures and Experimental Conditions for ATPase Assays**

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Assay</th>
<th>Temperature</th>
<th>Incubation (min)</th>
<th>Final concentration (mM) (total volume, 2 ml)</th>
<th>Final protein concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myofibril</td>
<td>Basic ATPase</td>
<td>25°C</td>
<td>5</td>
<td>Imidazole, 10; MgCl₂, 3.5; azide, 5; Tris-ATP, 2.5; pH 7.0; ionic strength, 0.074</td>
<td>0.06-0.11</td>
</tr>
<tr>
<td></td>
<td>Ca2⁺-activated</td>
<td>As above + CaCl₂, 0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Mg2⁺-activated</td>
<td>30°C</td>
<td>10</td>
<td>Sucrose, 250; Tris, 100 (pH 7.4); MgCl₂, 5.0; Tris-ATP, 2.5</td>
<td>1.7-2.0</td>
</tr>
<tr>
<td></td>
<td>Ca2⁺-activated</td>
<td>As above + CaCl₂, 5.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcoplasmic reticulum</td>
<td>Basic ATPase</td>
<td>35°C</td>
<td>10</td>
<td>KCl, 100; Tris-maleate (pH 6.8); 20; MgCl₂, 10; azide, 5; EGTA, 2; Tris-ATP, 0.2</td>
<td>0.05-0.1</td>
</tr>
<tr>
<td></td>
<td>Ca2⁺-activated</td>
<td>As above except no EGTA, + CaCl₂, 0.03, 0.12, or 0.24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2 Reaction Mixtures for Measurements of Calcium Accumulation by Mitochondria and Sarcoplasmic Reticulum with Dual Wavelength Spectrophotometry

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Measurement</th>
<th>Temperature</th>
<th>Final concentration of reagents (mM)</th>
<th>Final protein concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>&quot;Limited&quot; Ca(^{2+}) loading</td>
<td>30°C</td>
<td>Sucrose, 250; Tris-HCl buffer (pH 7.2), 10; murexide, 0.2; CaCl(_2), 0.12; sodium succinate, 50</td>
<td>0.7–1.0</td>
</tr>
<tr>
<td></td>
<td>&quot;Anion (phosphate)-assisted&quot; Ca(^{2+}) loading</td>
<td>As above except CaCl(_2), 0.36; phosphate, 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcoplasmic reticulum</td>
<td>Ca(^{2+}) accumulation</td>
<td>35°C</td>
<td>KCl, 100; Tris-maleate buffer (pH 6.8), 20; MgCl(_2), 10; murexide, 0.2; CaCl(_2), 0.03; Tris-ATP 0.2</td>
<td>0.2–0.4</td>
</tr>
<tr>
<td></td>
<td>Ca(^{2+}) accumulation with oxalate</td>
<td>As above except CaCl(_2), 0.12; oxalate, 5; Tris-ATP, 0.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ca Transport—Millipore Method (SR)

We measured calcium “uptake” by SR preparations (final protein concentration, 0.1 mg/ml), incubated at 35°C in a 5-ml reaction mixture containing 100 mM KCl, 20 mM Tris-maleate buffer, pH 6.8, 10 mM MgCl\(_2\), 5 mM potassium oxalate, and CaCl\(_2\) labeled with \(^{45}\)Ca\(^{2+}\) (Radiochemical Centre, Amersham).

The reaction was started by adding Tris-ATP (final concentration, 0.4 mM) and stopped by filtering a 1-ml sample 15 minutes later (0.45 μm diameter pore size HA Millipore), washing the filter (2 ml of 100 mM KCl, 20 mM Tris-maleate buffer, pH 6.8), dissolving the filter paper (Bray’s formula scintillation fluid), and measuring the calcium content of the trapped SR by scintillation spectrometry. The effect of caffeine on calcium “uptake” was studied by preincubation with 10 mM caffeine at three different initial calcium concentrations (30, 120, and 240 μM CaCl\(_2\)).

Direct Effect of Adding Caffeine to SR

The effect of adding caffeine to calcium-loaded SR vesicles was also studied directly, both by the Millipore technique with vesicles preloaded with calcium in the presence of oxalate, and by the murexide technique with vesicles preloaded in the absence of oxalate. In the Millipore experiments, SR was preincubated for 30 minutes in the reaction mixture (see above) with 30 μM CaCl\(_2\), 0.4 mM ATP, and the ATP-regenerating system. Two 5-ml samples were then centrifuged at 37,000 g, at 4°C, for 30 minutes, after which the resulting pellets were gently homogenized at 35°C with 5 ml of 100 mM KCl, 20 mM Tris-HCl buffer, pH 6.8, with and without 10 mM caffeine. These experiments were...
TABLE 3  Effect of Caffeine on Mitochondrial P/O Ratio, Oxygen Uptake (State 3 Respiration Rate), and Respiratory Control Index (RCI)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>10 mM caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/O ratio</td>
<td>2.96 ± 0.03</td>
<td>3.02 ± 0.01</td>
</tr>
<tr>
<td>Oxygen uptake n ats/mg protein per min</td>
<td>116.26 ± 5.18</td>
<td>107.24 ± 2.61</td>
</tr>
<tr>
<td>RCI</td>
<td>12.60 ± 0.50</td>
<td>11.34 ± 0.43</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE.

Conducted also with and without calcium (buffer normally contained 15 μM Ca, measured by atomic absorption spectrometry) by adding 30 μM CaCl2 or 2 mM ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) to the buffer. After 5 minutes, a 1-ml sample was passed through the Millipore filter and the calcium content of the filtrate (relative to the prefiltration total) was measured by scintillation spectrometry. In the murexide experiments, SR was preincubated similarly but for only 1.5 minutes. The reaction was followed by dual wavelength spectrophotometry after adding (in reservoir B) either 10 mM caffeine and 0.2 mM murexide, or 0.2 mM murexide alone, to SR preincubated (in reservoir A) for 1.5 minutes with 30 μM calcium and ATP with ATP-regenerating system.

Statistical Analysis

Results are given as mean ± standard error, compared by paired t-test, and regarded as significantly different where P < 0.05.

Results

Myofibrils

Caffeine did not alter basic or Ca2+-activated ATPase activity up to a concentration of 10 mM but did increase these at 20 mM* (P < 0.025) (Fig. 1).

Mitochondria

Caffeine (10 mM)* did not alter oxygen uptake (state 3 respiration rate) or P/O ratio with glutamate as substrate (Table 3), nor did it alter limited calcium loading analyzed in terms of total calcium accumulation and first order rate constant (Fig. 2). Caffeine did reduce the total amount of calcium accumulated in the presence of the permeant anion phosphate but did not alter the first order rate constant for this process (Fig. 3). It did not alter

* The study was concerned primarily with caffeine concentrations up to 10 mM, pilot experiments with 20 mM caffeine having given results, in each case, similar to those seen with 10 mM caffeine, with both mitochondrial and SR preparations.

FIGURE 1  Effect of caffeine on basic and Ca2+-activated ATPase of myofibril preparations (mean ± se, n = 6). The values indicated by asterisks are significantly different from control (P < 0.025).

FIGURE 2  Effect of caffeine on mitochondrial "limited" Ca2+ loading. A representative control dual wavelength spectrophotometric trace using murexide is shown above. The data summarized below show that caffeine altered neither the total Ca2+ bound nor the rate constant (mean ± se, n = 6).
transport into the mitochondrial matrix without influencing calcium binding or energy supply.

Sarcoplasmic Reticulum

Caffeine diminished total calcium accumulation but increased the rate constant for this process (Fig. 5). Caffeine also impaired calcium accumulation with oxalate, slowing the linear rate of calcium accumulation (Fig. 6). This slowing of calcium accumulation with oxalate became less marked at higher external calcium concentrations (Table 4). Caffeine (10 mM) did not alter sarcoplasmic reticulum basic ATPase activity either in the presence or absence of oxalate (Table 5). Caffeine significantly stimulated Ca²⁺-activated ATPase activity but only when the initial calcium concentration was 30 μM and not at higher calcium concentrations or in the presence of oxalate (Fig. 7).

In no case did the addition of caffeine cause any additional ("triggered") release of calcium beyond that predicted from the measurements of calcium transport in the presence of caffeine. Using the murexide technique, the addition of caffeine to SR,

\[
\Delta A = 0.011
\]

\[
\begin{array}{c}
\text{Co}^2+ \text{ACCUMULATED} \\
\text{RATE CONSTANT}
\end{array}
\]

\[
\text{Caffeine (mM)}
\]

\[
\text{Mg}^2+ \text{ATPase activity (associated with respiration)}
\]

\[
\begin{array}{c}
\text{BASIC (Mg}^2+) \text{ATPase} \\
\text{Ca}^2+ \text{ACTIVATED ATPase}
\end{array}
\]

\[
\text{Caffeine (mM)}
\]

\[
\begin{array}{c}
\text{Ca}^2+ \text{ACCUMULATION} \\
\text{RATE CONSTANT}
\end{array}
\]

\[
\text{Caffeine (mM)}
\]
FIGURE 6  Effect of caffeine on rate of Ca\(^{2+}\) accumulation in the presence of oxalate (initial Ca concentration, 120 \(\mu\)M). Representative traces are shown above (as in Fig. 2); C = control, 1 = 1 mM caffeine, 10 = 10 mM caffeine. The rates of Ca\(^{2+}\) accumulation during the linear phase summarized below are shown to be slowed by caffeine (mean ± SE, n = 4, P < 0.05 marked with asterisk).

actively loaded with calcium in the absence of oxalate, did not induce any obvious rapid release of calcium but simply resulted in a symmetrical change in the absorbance curve compatible with the other findings in this study. The effect of adding caffeine and murexide to unloaded SR (without ATP) was not different from that of adding murexide alone, indicating that caffeine did not cause any release of endogenous calcium from "unloaded" SR. Using the Millipore technique, it was shown that caffeine could increase calcium efflux from SR actively loaded with calcium in the presence of oxalate. Caffeine (10 mM) significantly increased the \(^{45}\)Ca loss after 5 minutes (22.1 ± 3.6% cf. 17.1 ± 3.4%, n = 7, P < 0.005) when external calcium concentration was very low (with EGTA), but not significantly when 30 \(\mu\)M CaCl\(_2\) was present in the buffer (18.0 ± 3.2% cf. 16.1 ± 3.7, n = 7, not significant), and the outward calcium gradient was therefore lower.

Discussion

Caffeine (1-10 mM) did not alter myofibrillar ATPase activity. At 20 mM, it increased both basic and calcium activated ATPase activity. The explanation for this observation is not clear and will need study with purer preparations of the individual contractile proteins. There is some physiological support for an action of caffeine on the function of

<table>
<thead>
<tr>
<th>Initial calcium concentration ((\mu)M)</th>
<th>nmol Ca accumulated/mg protein per 15 min</th>
<th>Control</th>
<th>Caffeine 10 mM</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>3146 ± 935</td>
<td>1285 ± 232</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>3996 ± 707</td>
<td>3221 ± 596</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>5686 ± 912</td>
<td>4542 ± 712</td>
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</tr>
</tbody>
</table>

Results in columns 2 and 3 are expressed as mean ± SE. n = 3.

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TABLE 5  Effect of Caffeine on SR Basic ATPase Activity

<table>
<thead>
<tr>
<th></th>
<th>nmol P(_i)/mg protein per 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>No oxalate</td>
<td>510 ± 64</td>
</tr>
<tr>
<td>With oxalate</td>
<td>1373 ± 154</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE. n = 6.
the contractile proteins in preparations supposedly isolated from membrane control.32

Caffeine (10 mM) impaired phosphate-assisted calcium transport into the matrix of isolated mitochondria but did not alter the more rapid process of calcium binding or the ability to supply energy (oxygen uptake, P/O ratio, Mg2+ ATPase activity) for this process.

Caffeine impaired calcium accumulation by isolated SR vesicles, both with and without oxalate, but paradoxically it could increase the rate constant for calcium accumulation and the Ca2+-activated ATPase activity. It has been reported previously for SR from skeletal muscle that caffeine can increase ATPase activity while not altering calcium uptake, or that it can leave ATPase unaltered while reducing calcium uptake, depending upon the experimental conditions.3 A similar dissociation of caffeine's effects on ATPase activity and calcium uptake has been reported for SR from cardiac muscle of rats and guinea pigs.18 Our data are thus in accord with these previous observations. We have further shown that dissociation can be related to the outward calcium concentration gradient, in that caffeine increases ATPase activity and inhibits calcium accumulation to a greater extent when the external calcium concentration is low, and to a smaller extent if the internal free calcium concentration is kept low by oxalate. These findings imply that caffeine exaggerates the passive efflux of calcium from SR vesicles when the outward gradient is high and thus that caffeine acts by making the SR membrane more permeable to calcium.

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