Effects of Adenosine 3':5'-Monophosphate-dependent Protein Kinase on Sarcoplasmic Reticulum Isolated from Cardiac and Slow and Fast Contracting Skeletal Muscles*

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Effects of cyclic adenosine 3':5'-monophosphate (cyclic AMP)-dependent protein kinase were studied in sarcoplasmic reticulum prepared from cardiac and slow and fast (white) skeletal muscle. Cyclic AMP-dependent protein kinase failed to catalyze phosphorylation of fast skeletal muscle microsomes as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cyclic AMP-dependent protein kinase was without effect on calcium uptake by these microsomes. Treatment of cardiac microsomes obtained from dog, cat, rabbit, and guinea pig with cyclic AMP-dependent protein kinase and ATP resulted in phosphorylation of a 22,000 dalton protein component in the amounts of 0.75, 0.25, 0.30, and 0.14 nmol of phosphorus/mg of microsomal protein, respectively. Calcium uptake by cardiac microsomes was stimulated 1.8- to 2.5-fold when microsomes were treated with cyclic AMP-dependent protein kinase. Protein kinases partially purified from bovine heart and rabbit skeletal muscle were both effective in mediating these effects on phosphorylation and calcium transport in dog cardiac sarcoplasmic reticulum. Slow skeletal muscle sarcoplasmic reticulum also contains a protein with a molecular weight of approximately 22,000 that can be phosphorylated by protein kinase. Phosphorylation of this component ranged from 0.005 to 0.016 nmol of phosphorous/mg of microsomal protein in dog biceps femoris. A statistically significant increase in calcium uptake by these membranes was produced by the protein kinase.

Increases in protein kinase-catalyzed phosphorylation of a low molecular weight microsomal component and in calcium transport by sarcoplasmic reticulum of cardiac and slow skeletal muscle may be related to the relaxation-promoting effects of epinephrine seen in these types of muscle. Conversely, the absence of a relaxation-promoting effect of epinephrine in fast skeletal muscle may be associated with the lack of effect of cyclic AMP and protein kinase on calcium transport by the sarcoplasmic reticulum of this type of muscle.

An effect of cyclic AMP-dependent protein kinase to increase calcium transport by cardiac sarcoplasmic reticulum

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1The abbreviations used are: cyclic AMP, adenosine 3':5'-monophosphate; protein kinase, adenosine 3':5'-monophosphate-dependent protein kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid.

suggests that this enzyme may mediate part of the mechanical response of the heart to catecholamines and other hormones and drugs that act to increase cyclic AMP levels within the myocardium (2-5). We have obtained evidence that protein kinase catalyzes the phosphorylation of a 22,000-dalton component of the cardiac sarcoplasmic reticulum isolated in vitro. This phosphorylation, which represents the formation of a phosphoester bond, is paralleled by stimulation of calcium uptake and Ca2+-activated ATPase activity. The increased rate of calcium uptake by the sarcoplasmic reticulum may account for the increased rate of relaxation of heart muscle which characterizes the cardiac response to epinephrine (6).

Like cardiac muscle, slow skeletal muscle also responds to epinephrine with an increased rate of relaxation (7-9). In fast (white) skeletal muscle, however, epinephrine characteristically produces a decrease in relaxation rate (7, 8). In view of the
different responses of these muscle types to epinephrine, we compare in the present communication the effects of protein kinase on sarcoplasmatic reticulum prepared from cardiac and slow and fast skeletal muscles. We present evidence that suggests that, unlike its effect in fast skeletal muscle, a relaxation-promoting effect of epinephrine in cardiac and slow skeletal muscle may be related to the ability of protein kinase to phosphorylate a 22,000-dalton component and stimulate calcium transport in sarcoplasmic reticulum obtained from these types of muscle.

EXPERIMENTAL PROCEDURE

Materials

Fresh muscle tissue was obtained from dogs and cats anesthetized with pentobarbital, or guinea pigs and New Zealand white rabbits killed by a sharp blow to the base of the skull.

Microsomes consisting mainly of fragmented sarcoplasmic reticulum were prepared from cardiac ventricles or skeletal muscle by the method of Harigaya and Schwartz (10), modified as described previously (3). The second head of the biceps femoris of the dog and rabbit soleus were chosen as slow skeletal muscle. Each preparation of soleus muscle microsomes was derived from muscle of four to six rabbits. The white-appearing proximal hind leg muscles of the rabbit were used as fast skeletal muscle (11); care was taken not to include semitendinosus, vastus intermedius, or gluteus maximus. Microsomes were also prepared from rabbit and cat tibialis anterior, which are also white skeletal muscle.

Bovine cardiac and rabbit skeletal muscle protein kinase used in the experiments shown in Fig. 1 were prepared and assayed as described previously (3). Bovine cardiac protein kinase used in all other experiments was prepared similarly except that it was concentrated to a final concentration of 5 to 10 mg/ml by means of an Amicon Model MC micro-ultrafiltration system with a PM 10 filter. Protein kinase activity was assayed in the same reaction mixture described previously (3) except that protein kinase concentrations were reduced to 10 ng/ml. Phosphoprotein was separated from unreacted ATP and breakdown products by the chromatographic method of Li and Felmly (12). The specific activity of the enzyme ranged from 1 to 7 nmol of phosphorus transferred/mg of enzyme/min.

Phosphorylation of Microsomes by Cyclic AMP-dependent
Protein Kinase

Procedure I—Microsomes (0.5 mg/ml) were incubated for 10 min at 25°C in 40 mM histidine-HCl (pH 6.8), 120 mM KC1, 5 mM ATP, 5 mM MgCl2, and either 1 µM cyclic AMP and varying amounts of protein kinase in histidine-HCl (pH 6.8) (final concentration 0.5 mM), or 0.5 mM histidine-HCl (pH 6.8) (control solution).

Procedure II—Microsomes (0.5 to 3.0 mg/ml) were incubated for 10 min at 25°C in 40 mM histidine-HCl (pH 6.8), 120 mM KC1, 25 mM NaF, 2.5 mM EGTA, 10 mM MgCl2, 0.5 mM [γ-32P]ATP (specific radioactivity greater than 7 × 10⁶ cpm/mmol), and either 1 µM cyclic AMP and 0.10 to 0.75 mg of protein/ml of bovine cardiac protein kinase contained in histidine-HCl (pH 6.8) (final concentrations, 0.04 to 0.20 mM), or control solution.

Determination of Phosphoprotein by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Microsomes were incubated as described under “Procedure II” for phosphorylation of microsomes. Reactions were terminated by the addition of 2 ml of ice-cold 10% trichloroacetic acid containing 0.1 M KH2PO4. Samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to “Procedure II” described in a previous communication (5). Procedures for electrophoresis were those of Weber and Osborn (13).

Assay for Calcium Uptake

Procedure A—Microsomes were preincubated according to “Procedure I” for phosphorylation of microsomes. At 10 min, aliquots of the reaction mixture were transferred to separate tubes containing 50 mM histidine-HCl (pH 6.8 to 7.0), 120 mM KC1, 2.5 mM Tris-oxide, 5 mM ATP, 5 mM MgCl2 and a *Ca2+-EGTA* buffer that gave a final ionized Ca²⁺ concentration of either 0.3 µM (125 mM CaCl2 and 1.058 mM EGTA, or 25 µM CaCl2 and 200 mM EGTA), or 0.75 µM (25 µM CaCl2 and 84 µM EGTA, or 125 µM CaCl2 and 487 µM EGTA) (14). Final microsome concentrations were 5 to 25 µg of protein/ml. Samples were taken and filtered through Millipore filters.

Procedure B—Microsomes (5 to 25 µg of protein/ml) were preincubated for 10 min in the complete reaction mixture described for “Procedure A” except that the *Ca2+-EGTA* buffer was omitted. At 10 min the calcium uptake reaction was started by addition of the *Ca2+-EGTA* buffer. Further details are given under “Procedure B” described in an earlier communication (5).

RESULTS

Fast Skeletal Muscle Microsomes—Treatment of sarcoplasmatic reticulum prepared from fast skeletal muscle of rabbit with cyclic AMP and protein kinase had no effect on the initial rate of calcium uptake when measured by Procedure A (Table I). Failure of fast skeletal muscle microsomes to exhibit the stimulation of calcium uptake seen with cardiac microsomes was noted when calcium uptake was measured at ionized Ca²⁺ concentrations between 0.3 and 1 µM. Decreasing the duration of the preincubation period to 1 min did not induce an increase in calcium uptake rate. The age of microsomal preparations was not a factor, since calcium uptake by microsomes either freshly prepared or aged for up to 9 days was not affected by cyclic AMP and protein kinase. In a single experiment, calcium uptake rates of microsomes prepared from the tibialis anterior of rabbit and cat were also not affected by protein kinase and cyclic AMP.

To determine whether fast skeletal muscle microsomes are isolated in a phosphorylated state, which could account for their insensitivity to protein kinase, microsomes were treated with either a commercially supplied alkaline phosphatase or a cardiac phosphoprotein phosphatase, then centrifuged and resuspended as usual. Neither phosphatase preparation inhibited the rate of calcium uptake, nor did protein kinase have any effect on calcium uptake by phosphoprotein phosphatase-treated microsomes. Similar negative findings were obtained

### Table I

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Calcium uptake</th>
<th>N</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.133 ± 0.007</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Protein kinase + cyclic AMP</td>
<td>0.129 ± 0.014</td>
<td>4</td>
<td>&lt;0.8</td>
</tr>
<tr>
<td>Boiled protein kinase + cyclic AMP</td>
<td>0.133 ± 0.014</td>
<td>3</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>Cyclic AMP</td>
<td>0.153 ± 0.015</td>
<td>3</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Protein kinase</td>
<td>0.141 ± 0.015</td>
<td>3</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Boiled protein kinase</td>
<td>0.139 ± 0.019</td>
<td>3</td>
<td>&lt;0.8</td>
</tr>
</tbody>
</table>

*Based on Student's t test for paired variates when compared with control value.*
when microsomes were incubated overnight at 2°C in the presence and absence of 5 mM MgCl₂, in order to activate any intrinsic phosphatase that may be associated with the microsomes.

No radioactivity attributable to protein kinase-catalyzed phosphorylation of microsomes was apparent after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fast skeletal muscle microsomes incubated with [γ-³²P]ATP, cyclic AMP, and bovine cardiac or rabbit skeletal muscle protein kinase (Fig. 1A). The single peak of radioactivity (Peak I) seen in this figure can be attributed to the cardiac protein kinase preparation, and corresponds to autophosphorylation of the 55,000-dalton cyclic AMP-binding subunit of cardiac protein kinase (15). An analogous peak of autophosphorylation was not observed for skeletal muscle protein kinase. No phosphorylation of skeletal muscle sarcoplasmic reticulum was observed when the period of incubation with cyclic AMP and protein kinase was varied between 0.5 and 60 min. Pretreatment of microsomes with cardiac phosphoprotein phosphatase, as described above, prior to their incubation with cyclic AMP and microsomes with cardiac phosphoprotein phosphatase, as described above, prior to their incubation with cyclic AMP and protein kinase, failed to result in protein kinase-catalyzed phosphorylation of microsomes.

Cardiac Microsomes—Microsomes prepared from dog heart, as previously reported (5), contain a 22,000-dalton component that is phosphorylated by cardiac protein kinase (Fig. 1B, Peak II). This component can also be phosphorylated by skeletal muscle protein kinase or the intrinsic protein kinase associated with the sarcoplasmic reticular membranes, as indicated by the small peak of radioactivity seen in the absence of added protein kinase (control).

Protein kinase-catalyzed phosphorylation of cardiac sarcoplasmic reticulum is seen in microsomes obtained from a variety of mammalian species (Table II). Treatment of cardiac microsomes obtained from dog, guinea pig, rabbit, and cat resulted in increased rates of calcium uptake. The percentage increases in calcium uptake rate and phosphorylation, as well as the absolute magnitude of the parameters shown in Table II, are not necessarily maximum, since optimum conditions were not defined for each measurement. In rabbit and guinea pig cardiac microsomes, as in those from the dog (5), virtually all protein kinase-catalyzed phosphorylation was associated with a microsomal component of approximately 22,000 daltons. However, in cat cardiac microsomes, significant additional phosphorylation was associated with a component of approximately 11,000 daltons in some, but not all, microsomal preparations. This latter peak of phosphorylation may be similar to a minor peak seen at the same location on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of dog cardiac microsomes (Fig. 1B, Peak III) (5).

**Slowed Skeletal Muscle Microsomes—Effects of protein kinase on microsomes derived from slow skeletal muscle were studied because these muscles, which are generally red in appearance (16), typically respond to β-adrenergic agonists with an acceleration of relaxation similar to that seen in the heart. Microsomes prepared from canine biceps femoris muscle, which has a low myosin ATPase activity (11, 17), characteristic of slow muscles (18), showed a statistically significant increase of approximately 23% in the initial rate of calcium uptake when measured after treatment of microsomes with cyclic AMP**.

### Table II

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein Kinase In-crease</th>
<th>Control</th>
<th>Protein Kinase In-crease</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphorylation</td>
<td>Calcium uptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nmol P/mg</td>
<td>fold</td>
<td>nmol Ca/mg/min</td>
<td>fold</td>
</tr>
<tr>
<td>Dog</td>
<td>0.08</td>
<td>0.75</td>
<td>9.4</td>
<td>0.04</td>
</tr>
<tr>
<td>Cat</td>
<td>0.06</td>
<td>0.25</td>
<td>4.2</td>
<td>0.05</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>0.01</td>
<td>0.14</td>
<td>14.9</td>
<td>0.05</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.07</td>
<td>0.30</td>
<td>4.5</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**Fig. 1.** Comparison of effects of different protein kinases and cyclic AMP on microsomes prepared from (A) rabbit fast skeletal muscle and (B) dog heart. Microsomes (2.0 mg/ml) were incubated in the presence and absence (x) of bovine cardiac (C) or rabbit skeletal muscle (D), protein kinase (0.45 mg/ml), and 1 μM cyclic AMP, together with 0.5 mM [γ-³²P]ATP. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described under "Experimental Procedure." Specific activity of the [γ-³²P]ATP was 1.83 x 10⁵ cpm/mmol. A total of 401 μg of skeletal muscle microsomal and 415 μg of cardiac microsomal protein was applied to the gels. The amount of phosphorylation found in the main peak of radioactivity was 0.08 nmol of phosphorus/mg of microsomal protein for control cardiac microsomes, 0.45 nmol of phosphorus/mg of microsomal protein in the presence of skeletal muscle protein kinase, and 0.75 nmol of phosphorus/mg in the presence of cardiac bovine protein kinase.
Protein Kinase and Calcium Transport

Table III
Effect of protein kinase and cyclic AMP on calcium uptake by slow skeletal muscle microsomes

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Calcium uptake (μmol Ca/min/mg protein)</th>
<th>Protein kinase + cyclic AMP</th>
<th>Increase</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine biceps femoris</td>
<td>0.262 ± 0.022 (7)</td>
<td>0.323 ± 0.033</td>
<td>23</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Rabbit soleus</td>
<td>0.145 ± 0.039 (6)</td>
<td>0.184 ± 0.051</td>
<td>27</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Based on Student's t test for paired variables.

AMP and protein kinase (Table III). The increase was abolished when boiled protein kinase was used. Aging of microsomes on ice for up to 3 days did not affect the percentage of stimulatory effect of protein kinase, although absolute rates of calcium uptake decreased gradually after the 2nd day after preparation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that a protein of approximately 22,000 daltons (Fig. 2, Peak II) was phosphorylated by the protein kinase. The extent of phosphorylation of this microsomal component ranged from 0.05 to 0.13 nmol of phosphorus/mg of microsomal protein in four independent experiments. A second peak (Fig. 2, Peak III) corresponded to a protein of approximately 11,000 daltons. In addition, a small peak of radioactivity (Peak I') was found on the gel and corresponded to a protein slightly larger in molecular weight than the phosphorylated protein attributable to bovine cardiac protein kinase (Peak I). The omission of 25 mM fluoride in the phosphorylation medium resulted in a slight reduction in phosphorylation, regardless of whether Procedure A or B was used.

Microsomes prepared from rabbit soleus muscle, which also has low myosin ATPase activity (11, 19), showed a statistically significant increase in calcium uptake rate when measured by Procedure A in the presence of protein kinase and cyclic AMP (Table III). A small but definite peak of phosphorylation was seen on the sodium dodecyl sulfate-polyacrylamide gel electrophoretograms of rabbit soleus muscle microsomes incubated in the presence of protein kinase and cyclic AMP (Fig. 3, Peak II). The average amount of phosphorylation as measured by the area of Peak II observed in electrophoretograms of four different preparations was 0.002 nmol of phosphorus/mg of microsomal protein in the absence of protein kinase and cyclic AMP, and 0.015 nmol of phosphorus/mg of microsomal protein in their presence. The increase in phosphorylation was statistically significant (P < 0.05). Included in Fig. 3 are electrophoretograms of rabbit cardiac and fast skeletal muscle microsomes in order to show the relative amounts of phosphorylation (see legend to figure).

Artifacts in Measurements of Calcium Uptake—During the course of our studies on effects of protein kinase on calcium uptake by microsomes prepared from different types of muscle, we became aware of two artifacts that may influence the apparent rate of calcium uptake. By a mechanism not understood, pretreatment of rabbit fast skeletal muscle microsomes with a partially purified preparation of protein kinase and cyclic AMP caused a statistically significant increase in the initial rate of calcium uptake when measurements were carried out in the presence of high (Procedure B), but not low (Procedure A), concentrations of protein kinase. This stimulatory effect was not dependent on the presence of cyclic AMP, and prior inactivation of the protein kinase preparation by boiling did not affect the apparent stimulation. The lack of α-P-labeling of fast skeletal muscle microsomes under these conditions confirms the fact that this effect was not due to membrane phosphorylation.

Effects of protein kinase on calcium uptake by cardiac and slow skeletal muscle sarcoplasmic reticulum, when measured by Procedure A (Tables II and III), were cyclic AMP-dependent, and were abolished when protein kinase was boiled. Stimulation of calcium uptake of dog cardiac microsomes by protein kinase was abolished by using boiled protein kinase, regardless of whether Procedure A or B was used.

A second artifact in the measurement of calcium uptake can arise from the previously reported effect of soluble proteins on the permeability of the Millipore filters used for the assay of calcium uptake (20, 21). The latter effect would produce an apparent decrease in the rate of calcium uptake when measured in the presence of soluble protein kinase. In all experiments...

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**Figure 2 (left).** Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of slow skeletal muscle microsomes prepared from canine biceps femoris. Microsomes (1.86 mg/ml) were incubated in the presence (○) and absence (○) of 0.97 mg/ml of protein kinase and 1 μM cyclic AMP; together with [γ-32P]ATP. Electrophoresis was carried out as described under "Experimental Procedure." A total of 186 μg of microsomal protein was applied per gel. The specific activity of the [γ-32P]ATP was 3.327 x 10^6 cpm/μmol. The amount of phosphorylation corresponding to Peak II on the gel is 0.084 nmol of phosphorus/mg of microsomal protein.

**Figure 3 (right).** Comparison of sodium dodecyl sulfate-polyacrylamide gel electrophoretograms of microsomes obtained from different types of rabbit muscle. Cardiac (×) and slow (○) and fast (○) skeletal muscle microsomal protein and 322 μg of cardiac muscle microsomal protein was applied to the gels. The specific radioactivity of [γ-32P]ATP was 2.29 x 10^6 cpm/μmol. The amount of phosphorylation of cardiac microsomes as estimated from the area of Peak II is 0.254 nmol of phosphorus/mg of microsomal protein.
reported in this study, care was taken that the amount of soluble protein did not reach levels that would give rise to this artifact.

**DISCUSSION**

Exposure of cardiac sarcoplasmic reticulum to cyclic AMP and protein kinase has been linked to an increased rate of calcium transport (2, 22, 23) and phosphorylation of a 22,000-dalton protein of the membrane (5, 24). Concomitant increases in calcium uptake and phosphorylation, when measured after treatment with cyclic AMP and protein kinase, may be demonstrated in cardiac microsomes obtained from a variety of mammalian species (Table I). By more rapidly decreasing the ionized calcium concentration present in the vicinity of the contractile proteins, an increased rate of calcium transport by the sarcoplasmic reticulum may, in the intact heart, account for an increased rate of relaxation, a classical effect of catecholamines on the heart (6, 25). Slow skeletal muscle, like cardiac muscle, also responds to epinephrine with an increase in the rate of relaxation (7–9). The increased rate of relaxation may similarly be related to the increase in rate of calcium transport that is observed after treatment of the sarcoplasmic reticulum with protein kinase and cyclic AMP (Table III), and to the presence of a 22,000-dalton component (Figs. 2 and 3) that is phosphorylated by protein kinase.

If phosphorylation of a 22,000-dalton protein is related to the increased rate of relaxation observed in response to epinephrine in both cardiac and slow skeletal muscle, one might expect not to find this protein in fast skeletal muscle where the relaxation-promoting effects of epinephrine are absent. Using a wide variety of experimental conditions, we have been unable to measure any protein kinase-catalyzed phosphorylation of fast skeletal muscle microsomes (Figs. 1 and 3), nor have we been able to show any effect of protein kinase on calcium transport in this type of muscle (Table I). The failure to demonstrate an effect of protein kinase on these microsomes could not be shown to be due to isolation of microsomes in a phosphorylated state, as treatment of microsomes with phosphoprotein phosphatases affects neither the rate of calcium uptake nor the ability of these microsomes to serve as substrate for the protein kinase.

The present studies suggest that a specific cellular response to hormonally triggered cyclic AMP formation may depend on the presence of specific substrates that can be phosphorylated by a variety of protein kinases. Thus, the relaxation-promoting effect of epinephrine may be related to the presence in cardiac and slow skeletal muscle of a 22,000-dalton protein whose phosphorylation is catalyzed by protein kinase, while its absence in fast skeletal muscle may be attributed to the absence of an analogous low molecular weight substrate for protein kinase. Whether an analogous low molecular weight phosphoprotein may mediate the well known relaxant effects of catecholamines in certain types of smooth muscle by a similar mechanism remains an interesting question for further investigation (26).

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