Adenosine 3',5'-Monophosphate-Dependent Membrane Phosphorylation

A POSSIBLE MECHANISM FOR THE CONTROL OF MICROSONAL CALCIUM TRANSPORT IN HEART MUSCLE

By Paul J. LaRaia and Eugene Morkin

ABSTRACT

The role of cyclic adenosine 3',5'-monophosphate (AMP) in the control of microsomal calcium ion (Ca$^{2+}$) transport was studied in microsomes prepared from rabbit heart. These cardiac microsomes contained intrinsic cyclic AMP-dependent protein kinase activity that phosphorylated serine residues in a microsomal protein component with a molecular weight of about 20,000 (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Intrinsic phosphoprotein phosphatase activity of the microsomal membranes resulted in rapid dephosphorylation of these residues. Microsomes phosphorylated in the presence of $1 \times 10^{-6}$M cyclic AMP exhibited enhanced Ca$^{2+}$ uptake. We conclude that reversible phosphorylation of microsomal membranes may be an important mechanism for regulation of microsomal Ca$^{2+}$ transport by cyclic AMP.

KEY WORDS protein kinase phosphoprotein phosphatase catecholamines contractility

Cyclic adenosine 3',5'-monophosphate (AMP) is believed to be an important mediator of the actions of catecholamines and certain other hormones on myocardial metabolism and contractility (1). The major mechanism whereby catecholamines regulate glycogenolysis in heart and skeletal muscle involves stimulation of a soluble cyclic AMP-dependent protein kinase (2, 3). Catecholamines also augment myocardial contractility, and a rapid rise in myocardial cyclic AMP has been shown to accompany or precede detectable alterations in contractility (4-7). The possibility that cyclic AMP mediates the effects of catecholamines through regulation of intracellular calcium has been examined by several investigators, since the contraction-relaxation cycle of muscle is thought to depend on changes in the intracellular concentration of free calcium ions (Ca$^{2+}$) (8, 9). Entman et al. (10) have reported that preincubation of cardiac microsomes with cyclic AMP enhances energy-dependent Ca$^{2+}$ uptake. However, other workers (11-13) have found no effect on cyclic AMP on microsomal Ca$^{2+}$ uptake except at high ($1 \times 10^{-6}$M) concentrations (14).

Prompted by the demonstration of intrinsic protein kinase activity in cardiac microsomes (15, 16), we have attempted to identify its endogenous substrate and to examine the role of phosphorylation as a possible mediator of the effects of cyclic AMP on microsomal Ca$^{2+}$ transport. The results indicate that a single membrane protein serves as the endogenous substrate for the enzyme and that phosphorylation of microsomal membranes enhances energy-dependent Ca$^{2+}$ uptake. Also, we have demonstrated (17) that microsomes contain intrinsic phosphoprotein phosphatase activity that cleaves phosphate from the microsomes in the presence of cyclic AMP. This evidence suggests that reversible phosphorylation of cardiac microsomes might be an important mechanism whereby catecholamines exert their positive inotropic action on the heart. The present study is a more extensive investigation of these preliminary findings (17).

Methods

Experiments were performed on adult male albino rabbits (approximately 2.5 kg) that were maintained on a diet of rabbit chow and water. The rabbits were killed by a blow to the head, and their hearts were rapidly excised and chilled in crushed ice. After removal of fat and connective tissue, approximately 3 g of ventricular muscle from each heart was cut into small pieces with

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scissors and placed in a plastic centrifuge tube containing 4 volumes of 4 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0. The tissue suspension was homogenized three times in an ice bath with a Polytron (Brinkman Instruments Co.) at a raseogen setting of 3 for 5 seconds with a rest interval of 15 seconds (18). The homogenate was centrifuged at 8,700 g for 30 minutes to remove mitochondria, nuclei, and cellular debris. The supernatant fluid was centrifuged at 37,000 g for 40 minutes. The pellet was resuspended in 5 ml of 0.6M KCl and 0.02M Tris-maleate buffer, pH 6.8. This suspension was centrifuged at 37,000 g for 40 minutes, and the supernatant fluid was discarded. The microsomal pellet was resuspended in 4 mM EDTA, pH 7.0, to give a final protein concentration of 2 mg/ml. The yield of microsomal protein was about 0.5–0.6 mg/g wet weight ventricular tissue.

The extent of mitochondrial contamination of the microsomal fraction was checked by determination of cytochrome oxidase activity (19). The activity per milligram of protein in this fraction was less than 3% of that in the mitochondrial fraction from 8,700 g supernatant fluid.

The supernatant fluid was determined by liquid scintillation counting using Aquasol.

Phosphorylation of Microsomal Protein.—Microsomes were phosphorylated in 200 ml of a solution containing 0.05M sodium glycerol phosphate buffer, pH 7.0, 0.01M magnesium acetate, 10-3M adenosine triphosphate (ATP) at a final specific activity of 5 μC/mole, 0.5 mM ATP, and 50 μg of microsomal protein. The reaction was initiated by the addition of ATP and terminated by the addition of an equal volume of ice-cold 7.5% trichloroacetic acid. The pellet was washed three times at 0°C by resuspension in 0.1 ml of 1N NaOH and precipitation with 2 ml of 5% trichloroacetic acid. Afterwards, the pellet was dissolved at room temperature in 0.1 ml of 1N NaOH, and the radioactivity was determined by liquid scintillation counting using Aquasol.

Dephosphorylation of Microsomal Protein.—Phosphoprotein phosphatase activity was determined on microsomes that had been phosphorylated in the presence or the absence of 1 x 10-8M cyclic AMP as described in the preceding section. At the end of the incubation, the tubes were transferred to an ice bath, and 100 nmoles of disodium-ATP in 50 μliters of 4 mM EDTA was added to each tube. After mixing, the tubes were incubated at 30°C for 1–5 minutes, and the incubation was terminated by the addition of an equal volume of ice-cold 7.5% trichloroacetic acid. In some experiments, phosphorylated microsomes were centrifuged at 37,000 g for 30 minutes and resuspended at the same protein concentration in 0.02M Tris-maleate buffer, pH 7.0, prior to determination of the cleavage of labeled phosphate.

Release of 32P-Orthophosphate from Labeled Microsomes.—About 100 μg of phosphorylated microsomes containing about 300–600 counts/min of 32P was suspended either in 1 ml of a solution containing 0.8M hydroxylamine in 0.01M sodium acetate buffer, pH 5.3, or in 0.5M NaOH. Control samples were suspended in 1 ml of a solution containing 0.8M NaCl in 0.01M sodium acetate buffer, pH 5.3. After 10 minutes of incubation at 30°C, 0.5 ml of ice-cold 50% trichloroacetic acid was added and the tubes were centrifuged at low speed for 10 minutes. The radioactivity contained in the pellets and the supernatant fluid was determined by liquid scintillation counting using Aquasol.

Acid Hydrolysis.—Microsomes phosphorylated using 32P-ATP in the presence of the absence of cyclic AMP were hydrolyzed in 2N HCl at 100°C for 6 hours. The hydrolysate was dried in a stream of nitrogen and dissolved in a formic acid-acetic acid buffer, pH 1.8, for electrophoresis. Phosphoserine counts were corrected for destruction during hydrolysis (about 40%) by analysis on an amino acid analyzer (Phoenix) of a solution containing a known amount of phosphoserine that had been hydrolyzed under the same conditions.

Incubation with Pepsin.—About 1.7 mg of denatured microsomal protein that had been phosphorylated in the presence or the absence of cyclic AMP was incubated with pepsin (enzyme-substrate ratio 1:5) in 0.5 ml of 0.1N formic acid at 37°C. After 1 hour the digest was chilled in ice and centrifuged. The supernatant fluid, which contained more than 90% of the counts in the undigested material, was lyophilized and redissolved in electrophoresis buffer.

Gel Electrophoresis.—The method of Weber and Osborn (20) was used to determine the molecular weight of the phosphorylated microsomal protein. About 40 μg of microsomal protein was solubilized by heating for 5 minutes at 90°C in a mixture of 3% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol in 0.05M sodium phosphate buffer. The samples were subjected to electrophoresis at 7 ma/tube in 5 or 10% polyacrylamide gels that were 0.1M in phosphate buffer, pH 7.0, and contained 0.1% SDS. The electrophoresis buffer was 0.1M in phosphate buffer, pH 7.0, and contained 0.1% 2-mercaptoethanol and 0.1% SDS. After electrophoresis, gels were stained overnight with 0.25% Coomassie blue in 50% methanol and 9% acetic acid. Excess stain was removed by soaking, first in a mixture containing 50% methanol and 9% acetic acid and then in 7.5% acetic acid. Ovalbumin, trypsin, and ribonuclease A were used as markers for determination of molecular weight.

Autoradiographs were prepared from 5% SDS-polyacrylamide gels as previously described (17). To determine the position of the radioactive membrane protein in 10% SDS-polyacrylamide gels, the gels were frozen and sliced at 1-mm intervals using a mechanical slicer. The slices were placed in scintillation vials and solubilized by heating in 1 ml of Protosol at 40°C for 16 hours. Then 10 ml of Aquasol was added and the vials were counted in a liquid scintillation counter (Nuclear Chicago Mark I).

High-Voltage Electrophoresis.—Electrophoresis of acid hydrolysates and peptic digests of phosphorylated microsomes was performed by applying samples dissolved in electrophoresis buffer to Whatman 3MM paper. Electrophoresis was carried out at 2,500 v for 90 minutes in a buffer containing 8% formic acid and 0.9% acetic acid, pH 1.8, using a flat-plate apparatus (Savant). Two-dimensional peptide "maps" of peptic digest were performed by electrophoresis in the first dimension in formic acid-acetic acid buffer, pH 2.0. After drying, electrophoresis was performed in the second dimension using pyridine-acetic acid buffer, pH 6.5. Buffers for peptide maps were prepared as described by Ambler (21). Autoradiography of one- and two-dimensional electrophoretograms was performed for 5–14 days using medical X-ray film.

Microsomal Calcium Uptake and Binding.—Mi-
Microsomes phosphorylated in the presence or the absence of cyclic AMP were centrifuged at 37,000 g and resuspended in 1 ml of reaction mixture consisting of 0.02 M Tris-maleate buffer, pH 6.8, 0.01 M MgCl₂, 0.10 M KCl, 0.005 M sodium oxalate, 100 μM CaCl₂ containing 0.05 μc of °Ca, and 75–125 μg of membrane protein. The mixture was incubated for various periods in the presence or the absence of 2 mM Tris-ATP. At the termination of the incubation, the preparation was passed through a Millipore filter (HA 0.45μ) as described previously (18).

Calcium binding by microsomes was measured using the same reaction mixture as that used for determination of Ca²⁺ uptake, except that the CaCl₂ concentration was 1 × 10⁻⁶ M and sodium oxalate was omitted from the medium. The incubations were carried out for various time periods in the presence or the absence of 2 mM Tris-ATP. The reaction was terminated by passage of the medium through a Millipore filter.

Results

Microsomal Phosphorylation.—The time course of microsomal membrane self-phosphorylation is shown in Figure 1. Phosphate incorporation generally was linear for less than 1 minute in the presence or the absence of 1 × 10⁻⁶ M cyclic AMP. However, further addition of ATP to the mixture returned the reaction to the initial rate, suggesting that hydrolysis of ATP by microsomal adenosine-triphosphatase (ATPase) probably was responsible for limiting phosphate incorporation. An incubation time of 30 seconds was chosen for the standard assay of microsomal protein kinase activity. The microsomal enzyme also was active when histone was used as the substrate, but it was not active when casein was the substrate (Table 1).

Enzyme Concentration.—Under standard assay conditions phosphorylation of endogenous substrate was proportional to the amount of microsomal protein kinase in the range of 25 to 75 μg protein/tube (125 to 375 μg/ml) both in the presence and the absence of 1 × 10⁻⁶ M cyclic AMP (Fig. 2).

pH Optima.—The effect of pH on microsomal self-phosphorylation in the presence or the absence of 1 × 10⁻⁶ M cyclic AMP is shown in Figure 3. Maximum activity in the presence of cyclic AMP occurred at about pH 7.0. In the absence of cyclic AMP, the protein kinase activity was much lower and the pH curve showed a rather broad maximum.

**TABLE 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Basal Activity (pmoles P/mg min⁻¹)</th>
<th>Cyclic AMP Activity (pmoles P/mg min⁻¹)</th>
<th>Histone Activity (pmoles P/mg min⁻¹)</th>
<th>Cyclic AMP + histone Activity (pmoles P/mg min⁻¹)</th>
<th>Casein Activity (pmoles P/mg min⁻¹)</th>
<th>Cyclic AMP + casein Activity (pmoles P/mg min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone</td>
<td>124 ± 2</td>
<td>300 ± 5</td>
<td>76 ± 3</td>
<td>135 ± 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td></td>
<td></td>
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</table>

Assays were performed as described in Methods. Histone and casein were added at a final concentration of 600 μg/200 μliters. Values are means ± 1 SD for three determinations.
Phosphorylation of various amounts of microsomal protein in the presence (solid circles) or the absence (open circles) of $1 \times 10^{-5}$ M cyclic AMP. Incubation conditions are described in Methods. Values are means ±1 SD for eight determinations.

**Nucleotide Effects.**—The effects of cyclic AMP and cyclic guanosine 5'-monophosphate (GMP) on stimulation of microsomal membrane self-phosphorylation are shown in Figure 4. Maximum stimulation occurred at $1 \times 10^{-4}$ M cyclic AMP and at $1 \times 10^{-3}$ M cyclic GMP (Fig. 4A). Even at the highest cyclic GMP concentrations, full activation of the enzyme was not achieved. Addition of $1 \times 10^{-6}$ to $1 \times 10^{-4}$ M 5'-AMP did not stimulate phosphate incorporation above the basal level (not shown). Simultaneous addition of both cyclic nucleotides caused only a slight additive enhancement of phosphate incorporation into microsomal protein (Fig. 4B).

**Characterization of Phosphorylated Membrane Component.**—Extraction with an ethanol-ether solution (1:1, v/v) of trichloroacetic acid-precipitated microsomes that had been phosphorylated in the presence of cyclic AMP did not release any radioactivity, demonstrating that the substrate was not lipid. Since acyl phosphate intermediates involved in microsomal Ca$^{2+}$ transport might have been responsible for the observed
"P incorporation, microsomes phosphorylated in the presence of cyclic AMP were treated with hydroxylamine. Lipmann and Tuttle (22) have shown that under conditions such as those used in these experiments (pH 5.3 and incubation at 30°C) the nucleophilic attack by hydroxylamine is fairly specific for carboxylic acid anhydrides. Figure 5 demonstrates that the majority of the counts produced by cyclic AMP addition was resistant to incubation with hydroxylamine. However, incubation with NaOH, which cleaves phosphoester bonds (17), resulted in loss of over 90% of the counts. Measurement of radioactivity in strips from an electrophoretogram of phosphorylated microsomes that had been hydrolyzed for 6 hours in 2N HCl revealed that over 80% of the increase in "P incorporation stimulated by cyclic AMP could be accounted for as phosphoserine. Labeled phosphothreonine could not be detected in these electrophoretograms.

Identification of the Phosphorylated Protein.—Basal and cyclic AMP-stimulated microsomes contained radioactive material of low molecular weight which migrated with the tracking dye. However, only cyclic AMP-stimulated microsomes contained an additional radioactive component that corresponded to a faintly staining band in the stained gel. The molecular weight of this phosphoprotein was determined to be approximately 20,000 in 10% SDS-polyacrylamide gels (Fig. 6).

Phosphorylated Peptide.—Figure 7 shows an autoradiograph of a two-dimensional map of a 1-hour peptic digest of microsomes phosphorylated in the presence of cyclic AMP. Only a single strongly radioactive peptide and two weakly radioactive peptides were seen. This result suggests that phosphorylation might be limited to either a single serine residue or serine residues occurring in similar amino acid sequences, giving rise to a limited number of peptides.

Phosphoprotein Phosphatase.—The ability of cardiac microsomal phosphoprotein phosphatase to dephosphorylate endogenous microsomal substrate is shown in Figure 8. Microsomes phosphorylated in the presence or the absence of 1 x 10^-4 M cyclic AMP were assayed for phosphatase activity after addition of excess unlabeled ATP to prevent further isotope incorporation. Rapid cleavage of labeled phosphate from the cyclic AMP-stimulated microsomes occurred immediately on addition of excess nonradioactive ATP. The alkali-labile phosphate content of phosphorylated microsomes was about 175 pmoles/mg protein. Approximately half of this phosphate was removed in the first 3 minutes of incubation. There was no significant cleavage of phosphate in the basal prepa-
To test the possibility that the increase in Ca$^{2+}$ uptake resulted from a nonspecific stabilizing action of nucleotides on microsomal membranes (12), microsomes were preincubated for 30 seconds in either $1 \times 10^{-4}$M 5'-AMP or $1 \times 10^{-4}$M cyclic GMP. In these experiments, which also are shown in Figure 9, no difference in Ca$^{2+}$ uptake was noted between control and nucleotide-treated microsomes.

Table 2 demonstrates the results obtained for Ca$^{2+}$ binding to cardiac microsomes prephosphorylated in the presence or the absence of $1 \times 10^{-6}$M cyclic AMP. No significant difference was noted between the basal and the cyclic AMP-treated preparations.

**Discussion**

The results indicate that cardiac microsomes contain intrinsic cyclic AMP-dependent protein kinase activity that can phosphorylate exogenous histones or an endogenous membrane component. Microsomes phosphorylated in the presence of cyclic AMP exhibit transient enhancement of Ca$^{2+}$ uptake. Also, cardiac microsomes contain an intrinsic phosphoprotein phosphatase capable of removing phosphate from microsomes phosphorylated in the presence of cyclic AMP. Thus, the...
Figure 9

**Time course of energy-dependent Ca**<sup>2+</sup> uptake by microsomes phosphorylated in the presence of 1 × 10<sup>-4</sup> M cyclic AMP (solid diamonds), 1 × 10<sup>-4</sup> M cyclic GMP (open circles), or 1 × 10<sup>-4</sup> M 5'-AMP (solid circles). Values are means ± 1 SD for 6-12 determinations. The shaded area represents the range (±2 SD) of Ca**<sup>2+</sup> uptake values for microsomes phosphorylated without cyclic nucleotides or 5'-AMP (12 determinations).

### Table 2

**Calcium Binding by Rabbit Cardiac Microsomes**

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>Control</th>
<th>Cyclic AMP</th>
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<tbody>
<tr>
<td>30</td>
<td>28 ± 7</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>60</td>
<td>51 ± 10</td>
<td>47 ± 9</td>
</tr>
</tbody>
</table>

Assays were performed as described in Methods. Values are means ± 1 SD for eight determinations.
prolonged augmentation of microsomal Ca\(^{2+}\) uptake described is difficult to reconcile with a control system capable of rapidly modulating Ca\(^{2+}\) uptake. More work will be required to clarify the role of soluble vs. membrane-bound protein kinase in the control of myocardial function.

Membrane-bound phosphoprotein phosphatases have been identified in several tissues (24, 32, 33). In these tissues, as well as in the heart, the combined activities of membrane cyclic AMP-dependent kinases and membrane phosphoprotein phosphatases provide a dynamic regulatory system for the phosphate content of membrane proteins which is independent of the synthesis or the degradation of membrane subunits. In a subcellular membrane fraction from brain, the rate of dephosphorylation has been reported to be the limiting step in the turnover of protein-bound phosphate (24). The biological events which lead to dephosphorylation are poorly understood. De Lorenzo and Greengard (33) have shown that activation by cyclic AMP of a membrane-bound phosphoprotein phosphatase from toad bladder might play a role in modulation of the physiological effects of antidiuretic hormone on transport of water, sodium, or both in this tissue. The effect of cyclic AMP on cardiac microsomal phosphoprotein phosphatase is unknown presently.

Since the original suggestion by Rall and Sutherland (34) that cyclic AMP is involved in the glycogenolytic effect of epinephrine in the heart, considerable evidence has accumulated to suggest that cyclic AMP might also be involved in the positive inotropic response to catecholamines (see review by Epstein et al. [35]). The mechanism by which this effect is mediated has remained unclear. The earlier suggestion that epinephrine might act through activation of phosphorylase no longer seems likely (36, 37), and experiments which appeared to show a direct effect of cyclic AMP on the superprecipitation of actomyosin could not be confirmed (38). Since the importance of Ca\(^{2+}\) in the contractile process has become generally recognized (8, 9), efforts have been made to implicate cyclic AMP in the control of microsomal Ca\(^{2+}\) transport. However, the results of these studies (10–14) have been inconclusive.

The present results can be used to formulate a mechanism whereby catecholamines can modulate intracellular Ca\(^{2+}\) concentration via cyclic AMP-dependent microsomal membrane phosphorylation. Epinephrine and other catecholamines interact with the cell membrane to stimulate adenyl cyclase to produce cyclic AMP which, in turn, activates microsomal protein kinase. The activation of microsomal protein kinase results in phosphorylation of a specific membrane protein involved in the regulation of Ca\(^{2+}\) transport. Modulation of the state of phosphorylation of this protein by membrane protein kinase and membrane phosphoprotein phosphatase regulates microsomal Ca\(^{2+}\) metabolism. Alterations in microsomal Ca\(^{2+}\) transport regulate myocardial contractility by varying the availability of free Ca\(^{2+}\) to the contractile proteins. Several features of the proposed mechanism need further elaboration. An essential feature of the proposed mechanism is that microsomes phosphorylated in the presence of cyclic AMP exhibit enhanced Ca\(^{2+}\) uptake. It is now generally accepted that the contraction of heart and skeletal muscle requires the presence of Ca\(^{2+}\) in addition to Mg\(^{2+}\) and ATP (8, 9). The relaxing effect of microsomes is explained by their ability to lower the Ca\(^{2+}\) concentration below 1 x 10\(^{-6}\) M. Kinetic studies of the interaction of Ca\(^{2+}\) binding in the absence of oxalate or other anions that precipitate Ca\(^{2+}\) appear to reflect binding of Ca\(^{2+}\) to specific receptor sites made accessible for Ca\(^{2+}\) by ATP and Mg\(^{2+}\) (39). On the other hand, Ca\(^{2+}\) uptake measured in solutions containing oxalate reflects the transport of large quantities of Ca\(^{2+}\) into microsomal vesicles where the ion precipitates as calcium oxalate (40). This transport process is active, and it derives its energy from hydrolysis of ATP; the process might not involve the high-affinity Ca\(^{2+}\) binding sites. The present results indicate that stimulation of intrinsic microsomal protein kinase enhances the process of Ca\(^{2+}\) uptake but not Ca\(^{2+}\) binding. Increased Ca\(^{2+}\) uptake might be linked directly to stimulation of contractility. As proposed by Gertz et al. (41), stimulation of Ca\(^{2+}\) uptake during cardiac relaxation might allow more Ca\(^{2+}\) to be released during subsequent beats to the contractile proteins, thereby augmenting myocardial contractility.

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