Adenosine 3':5'-Monophosphate-dependent Protein Kinase-catalyzed Phosphorylation Reaction and Its Relationship to Calcium Transport in Cardiac Sarcoplasmic Reticulum

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SUMMARY

A rapid, manyfold increase in phosphorylation of cardiac microsomes consisting primarily of sarcoplasmic reticulum was seen when these membranes were incubated in the presence of a bovine cardiac adenosine 3':5'-monophosphate (cyclic AMP)-dependent protein kinase (protein kinase) and cyclic AMP. Over 85% of the 32P associated with membrane protein under similar conditions was identified as phosphoserine and phosphothreonine. A less marked increase in phosphoprotein formation was observed when cardiac microsomes were incubated in 1 μM cyclic AMP in the absence of added protein kinase. This could be attributed to the presence of an endogenous protein kinase. When cardiac microsomes were incubated with protein kinase alone, phosphorylation also was enhanced, finally reaching the level seen with cyclic AMP and added protein kinase. The increased phosphorylation induced by protein kinase alone was attributable to the presence of an adenylate cyclase in the microsomal preparation. Epinephrine could be shown to stimulate both adenylate cyclase and phosphorylation of cardiac microsomes.

Protein kinases prepared from both bovine cardiac or rabbit fast skeletal muscle catalyzed the formation of microsomal phosphoprotein. The extent of phosphoprotein formation correlated closely with the increment in the stimulation of the rate of calcium uptake by cardiac microsomes when concentrations of either protein kinase were varied, and the relationship between phosphoprotein formation and stimulation of calcium transport was independent of the source of the protein kinase. These data suggest the existence of a functional relationship between cardiac microsomal phosphorylation and an increased rate of calcium transport.

The early finding of Sutherland and Rall (4) that cellular effects of epinephrine are expressed through the action of cyclic AMP has been followed by a number of studies aimed at deciphering the biochemical sequence of events triggered by catecholamines, polypeptide hormones, and other physiological "messengers." Subsequently, others have found that cyclic AMP activates the enzyme protein kinase by combining with the binding subunit of this enzyme, thereby causing the dissociation of the binding subunit-cyclic AMP complex from the catalytic subunit of the enzyme (5-8). Protein kinases have been identified in a variety of mammalian tissues (9); several, including those from heart and skeletal muscle, have been purified and characterized (10, 11). The substrate specificity of the muscle protein kinases, like that of other protein kinases, appears to be broad as they can catalyze the phosphorylation of several protein substrates.

Information on the molecular nature of protein kinases is rapidly expanding (12) but relatively little is known about their natural protein substrates. Several protein components of smooth, skeletal, and cardiac muscle that were co-purified with soluble protein kinase from these sources were shown to be phosphorylated but were not further identified (13). Walsh et al. (14), however, have related phosphorylation of phosphorylase kinase by one of the protein kinases, phosphorylase kinase, to activation of glycogenolysis. Huttunen et al. (15) have shown that phosphorylation of a purified lipase preparation is correlated with lipase activation. Many examples of protein kinase-catalyzed membrane phosphorylation have been documented (16-21), but in most cases the identification of an alteration in a specific cell function as a direct consequence of protein kinase-catalyzed phosphorylation has not been possible.

1 The 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.

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Our recent finding that calcium uptake by isolated membranes of cardiac sarcoplasmic reticulum is stimulated by cyclic AMP-dependent protein kinase (22) permits the study in vitro of a sequence of biochemical reactions triggered by cAMP and resulting in a measurable effect on calcium transport. In the heart, where changes in the movements of calcium within the myocardial cell probably play a role in the regulation of physiological function, changes in the ability of sarcoplasmic reticulum to transport calcium are probably related directly to the control of the contractile properties of the intact heart (23). In the present communication, we present evidence that protein kinase-catalyzed phosphoprotein formation in a preparation of dog cardiac microsomes consisting mainly of sarcoplasmic reticulum is related to this stimulation of calcium transport.

**EXPERIMENTAL PROCEDURE**

**Materials**

Frozen bovine hearts (2 to 4 pounds) were supplied by Pel-Freez Biologicals, Inc., Rogers, Ark. $[^{32}P]ATP$ and $[^{3}P]ATP$ tetra (triethylammonium) salts, were obtained from New England Nuclear, Boston, Mass., with specific activities of 20 to 100 mCi per pmole and 5 to 10 mCi per pmole, respectively. $^4{\text{CaCl}}_2$ ($\sim 2$ mCi per pmole) also was supplied by New England Nuclear. Sevquanal grade $6\times$ hydrochloric acid was obtained from Pierce Chemical Company, Rockford, Ill. Polyethyleneimine-imregnated sheets (Polygram CEL 300 PEL, 20 × 20 cm) were obtained from Brinkmann Instruments, Inc., Westbury, N. Y. DEAE-cellulose (Whatman DE-52) was supplied by H. Reeve Angel, Inc., Dallas, Texas. Special enzyme grade ammonium sulfate, purified by the method of Lowry et al. (26) with bovine serum albumin as a standard. Adenylic and inosinic acid grade.

**Preparation of Cardiac Microsomes**

Canine cardiac microsomes, which consist mainly of sarcoplasmic reticulum, were prepared by the method of Harigaya and Schwartz (25) modified in that sodium azide was omitted and homogenization was carried out in a Waring Blender. For most experiments in which phosphorylation of microsomes by bovine cardiac protein kinase was measured, microsomes were used within 2 days after preparation as aging reduced phosphorylation slightly. In all other experiments, only microsomes prepared on the same day were used. Protein concentrations were measured by the method of Lowry et al. (26) with bovine serum albumin as a standard.

**Preparation of Protein Kinases**

Cardiac protein kinase was purified through the DEAE-cellulose chromatography step according to the method of Miyamoto et al. (27) from frozen bovine hearts that were stored at −20° prior to use. Skeletal muscle protein kinase was prepared from the white appearing back and hind limb muscles of rabbits. Care was taken not to include gluteus, semitendinosus, gastrocnemius, or soleus muscles. The enzyme was partially purified by the method of Watase et al. (28). The appropriate fractions containing protein kinase activity were dialyzed against 5 mm histidine-HCI buffer (pH 6.8) and could be stored in small aliquots at −20° for 1 to 2 months without detectable loss of activity.

**Specific Activity and Standardization of Protein Kinases**

The specific activities of skeletal and cardiac protein kinases were determined by measuring the incorporation of $^{32}P$ from $[^{32}P]ATP$ into histone. The reaction mixture (0.2 ml total volume) consisted of 40 mm histidine-HCl buffer (pH 6.8), 0.12 M KCl, 5 mm MgCl$_2$, 2.5 mm $[^{32}P]ATP$ (0.5 μCi per sample), 1.0 mg of histone per ml, 1 μM cyclic AMP, and 0.025 mg of cardiac protein kinase per ml or 0.05 mg skeletal protein kinase per ml. Incubation for 5 min at 25°. The amount of acid-precipitable phosphoprotein formed was determined by the method of Kuo and Greenberg (29). Under these assay conditions, the cardiac cyclic AMP-dependent protein kinase activity of different preparations varied from 0.15 to 0.61 nmole per mg of enzyme protein per min for cardiac protein kinase and 0.11 to 0.27 nmole per mg of enzyme protein per min for skeletal muscle protein kinase. Enzyme activity in the absence of cyclic AMP was negligible. The average phosphorylation of a single preparation of microsomes by 0.1 mg per ml of each of five different preparations assayed under standard conditions was 1.32 ± 0.07 (S.E.) nmole per mg of microsomal protein per 5 min.

**Microsomal Phosphorylation Assay**

Procedure I—The partial reaction mixture for phosphorylation of cardiac microsomes consisted of 40 mm histidine-HCl buffer (pH 6.8), 0.13 M KCl, 2.5 mm Tris-oxalate, a small amount of calcium-EGTA buffer (30) to maintain Ca$^{2+}$ concentration below 0.7 μM (EGTA = 84 μM, CaCl$_2$ = 25 μM), and 1 μM cyclic AMP and bovine protein kinase 0.1 mg per ml or both. Microsomes (0.5 mg per ml) were added after 5-min equilibration of the partial reaction mixture at 30°. The time of microsomes addition was taken as zero time. The temperature equilibration was continued for an additional minute at which time experimental reactions were started by the addition of a solution containing equimolar amounts of MgCl$_2$ and $[^3P]ATP$ (1 to 2 μCi per sample) to final concentrations of 3 mm. The incubation in a total volume of 0.2 ml was continued for 5 min. These conditions for incubation are referred to as "standard conditions" for phosphorylation measurements. In studies of the effects of Ca$^{2+}$ on microsomal phosphorylation, calcium-EGTA buffers (CaCl$_2$ = 10 mm, EGTA varied between 0.16 and 16 mm) were used to maintain Ca$^{2+}$ concentrations at 0.05, 0.2, 0.5, 1, 10, and 100 μM. Studies on the temperature dependence of phosphorylation were performed under standard conditions except that the protein kinase concentration was 0.05 mg per ml, the incubation time was 30 s, and 2.5 mm EGTA was used instead of calcium-EGTA buffer.

Reactions were stopped by addition of 2 ml of 10% (w/v) ice-cold trichloroacetic acid containing 0.1 mm KH$_2$PO$_4$ and 0.2 ml of 0.63% bovine serum albumin. After remaining on ice for 10 min, samples were centrifuged at 3° for 10 min at 1500 × g. The supernatant was aspirated and the pellets were dissolved in 0.1 ml of 0.5 M NaOH at room temperature. An additional 2 ml of the trichloroacetic acid solution was added, the centrifugation was repeated, and the pellets were washed with trichloroacetic acid three more times. The final pellets were dissolved in 0.1 ml of 0.5 M NaOH and transferred to plastic scintillation vials together with 13 ml of dioxane base scintillation fluid for counting. Total phosphorylation is expressed as nanomoles of $^{32}P$ transferred per mg of microsomal protein per 5 min unless otherwise indicated. In studies of the effects of Ca$^{2+}$ on microsomal phosphorylation and the temperature dependence of phosphorylation, the pellets also were treated with hydroxylamine (see below) which was included in the wash procedure after the first centrifugation of the acid precipitated microsomes.

Procedure II—Microsomes were incubated as described under Procedure I except that calcium-EGTA buffer and Tris-oxalate were omitted from the incubation medium. Protein kinase concentration was varied from 0 to 0.9 mg per ml, and reactions were carried out at 25° for 10 min. Hydroxylamine treatment was included in the trichloroacetic acid wash procedure.

**Adenylate Cyclase Assay**

Adenylate cyclase activity was measured under the same assay conditions used to determine microsomal protein kinase catalyzed phosphorylation according to Procedure I above with the following modifications. The total volume of the incubation mixture was 50 μl. Unlabeled cyclic AMP (0.05 mm) was included to protect labeled cyclic AMP from possible degradation by phosphodiesterase and MgCl$_2$ and ATP concentrations were 1 mm instead of 5 mm. Reactions were started by addition of microsomal protein and stopped by boiling for 3 min after addition of 50 μl of a solution containing 5 mm cyclic AMP and AMP. Radioactive cyclic AMP
was separated by polyethyleneimine cellulose thin layer chromatography according to the method Bär and Hechter (31) as modified by Tada et al. (32).

**Assay for Calcium Uptake**

**Procedure A—**In studies in which protein kinase-catalyzed microsomal phosphorylation was correlated with protein kinase-stimulated calcium uptake, microsomes were preincubated for 10 min in the same reaction medium described for Procedure II for measuring microsomal phosphorylation except that unlabeled ATP was used. At 10 min, an aliquot of the preincubation mixture was added to the assay medium for measuring calcium uptake. Final microsomal protein concentration was 0.05 mg per ml in 40 mM histidine-HCl buffer (pH 6.8), 0.12 M KCl, 2.5 mM Tris-oxalate, 5 mM ATP, 5 mM MgCl₂, and a 4Ca⁺-EGTA buffer that gave a final Ca⁺⁺ concentration of 0.75 mM (EGTA = 487 mM; CaCl₂ = 125 mM). Assays were performed as described previously (30).

Procedure B—Cardiac microsomes (0.048 mg per ml) were preincubated for 10 min at 25°C in 40 mM histidine-HCl buffer, pH 6.8, 0.12 M KCl, 2.5 mM Tris-oxalate, 5 mM ATP, 5 mM MgCl₂, with or without 1 μM cyclic AMP and 0.1 mg of cardiac or skeletal muscle protein kinase per ml. The calcium uptake reaction was started by addition of the 4Ca⁺-EGTA buffer (Ca⁺⁺ = 1 μM; EGTA = 391 μM; CaCl₂ = 125 mM).

**Characterization of Phosphorylated Protein**

**Stability in Hot Acid or Alkali—**Microsomes were incubated under standard assay conditions for phosphorylation and reactions were terminated as described above except that bovine serum albumin was omitted. Samples were centrifuged and 0.1 ml of 6 N NaOH or 10% trichloroacetic acid containing 0.1 M KH₂PO₄ was added to the microsomal pellets followed by heating at 90°C for 10 min. Control samples were left on ice in 0.1 ml of trichloroacetic acid solution for the same period of time. An additional 2 ml of ice-cold trichloroacetic acid solution were added together with 0.2 ml of 0.63% (w/v) bovine serum albumin. Samples were washed in the trichloroacetic acid solution three more times as described, including the washing in 0.5 N NaOH at room temperature and counted.

**Stability in Hydroxylamine—**Microsomes were incubated under standard conditions and reactions were terminated as described above. Samples were centrifuged and the pellets were treated with hydroxylamine as described by Lipmann and Tuttle (33). Treatment was terminated by the addition of 1 ml of 20% trichloroacetic acid, and successive washing of the acid-precipitable material with trichloroacetic acid solution and NaOH was carried out as described above.

**Identification of Phosphoserine and Phosphothreonine—**Microsomes were incubated for 10 min in the presence and absence of 1 μM cyclic AMP and 0.1 mg of protein kinase per ml or 1 μM cyclic AMP under standard conditions described above under "Procedure II" with the following modifications. The final concentrations of MgCl₂ and [γ-³²P]ATP were 0.5 mM and 25 mM sodium fluoride was present. The acid-precipitated protein was washed successively as described above except that bovine serum albumin was omitted. After the first wash with trichloroacetic acid the protein together with carrier phosphoserine and phosphothreonine was partially hydrolyzed for 1.5 hours in 6 N HCl at 110°C in vacuo. This time was chosen on the basis of preliminary experiments in which phosphoserine was hydrolyzed for varying periods of time and P₁ liberation was measured. After evaporation of HCl, the samples were taken up in 35 μl of H₂O and applied to Whatman No. 3MM chromatography paper (60 × 3 cm). Amino acids were separated by paper electrophoresis at 1500 volts for 1.5 hours in HV 5000 A Savant high voltage electrophoresis apparatus. The solvent consisted of 5% acetic acid, 0.5% pyridine (pH 3.5). Phosphoserine and phosphothreonine were located with a spray consisting of 1% ninhydrin in acetone. Radioactivity was located on the paper electrophoretograms with a Packard chromatogram scanner. The electrophoretograms were cut into pieces consisting of a broad region containing [³²P], and spots corresponding to [³²P] phosphoserine and [³²P] phosphothreonine. These pieces, along with 2 cm pieces of the remainder of the electrophoretograms, were counted by liquid scintillation spectrometry. Breakdown of microsomal phosphoserine and phosphothreonine during acid hydrolysis was estimated by subjecting known amounts of unlabeled phosphoserine and phosphothreonine to acid hydrolysis under the same conditions and measuring P₁ liberation by the method of Taussky and Shorr (34). Hydrolysis of unlabeled phosphoserine was unaffected by the presence of microsomal protein.

**RESULTS**

**Phosphorylation of Cardiac Microsomes by Bovine Cardiac Protein Kinase—**When microsomes were incubated with MgCl₂ and ATP in the absence of added cyclic AMP and protein kinase, slight but significant phosphorylation was seen (Fig. 1). In the presence of both cyclic AMP and protein kinase, phosphorylation was markedly stimulated, the initial rate of phosphorylation being increased 3- to 6-fold. In the presence of cyclic AMP alone, phosphorylation was increased slightly, less than 2-fold. Incubation with protein kinase alone caused little stimulation during the early part of the incubation, but after 10 to 20 min phosphorylation greatly exceeded that seen in the control reaction, eventually reaching the level obtained when cyclic AMP was added along with the protein kinase at the start of the reaction.

The dependence of protein kinase-catalyzed microsomal phosphorylation upon protein kinase concentration was studied in experiments where the microsomal protein concentration was maintained at 0.5 mg per ml and the protein kinase concentration was varied from 0 to 0.3 mg per ml (Fig. 2). In the presence of 1 μM cyclic AMP, microsomal phosphorylation increased with increasing protein kinase concentration until the protein kinase

![Fig. 1. Time course of microsomal phosphorylation. Cardiac microsomes (0.5 mg per ml) were incubated under standard conditions in the presence of 0.1 mg of bovine cardiac protein kinase per ml (●), 1 μM cyclic AMP (○), both (△), or neither (□). A typical experiment is shown in all figures unless otherwise indicated.](http://www.jbc.org/content/249/15/6168/F1.large.jpg)

![Fig. 2. Dependence of microsomal phosphorylation on concentration of bovine cardiac protein kinase. Cardiac microsomes (0.5 mg per ml) were incubated for 5 min under standard conditions in the presence of 1 μM cyclic AMP and increasing concentrations of protein kinase.](http://www.jbc.org/content/249/15/6168/F2.large.jpg)
Incubation under standard conditions for 5 min in the absence of ionized calcium maintained with calcium-EGTA buffers as described under “Experimental Procedure.”

Subsequent experiments were carried out at a protein kinase concentration of 0.1 mg per ml at a ratio of microsomal protein to protein kinase of 5:1.

The data shown in Fig. 2 have been corrected for phosphorylation attributable to the presence of added protein kinase. At the protein kinase concentration of 0.1 mg per ml, phosphorylation attributable to the protein kinase preparation was less than 8% of the total phosphorylation observed. The phosphorylation of microsomes was slower than that of the protein kinase preparation, so that the proportional phosphorylation of microsomes increased percentage-wise after prolonged incubation.

Protein kinase-catalyzed microsomal phosphorylation was found to be largely independent of Ca2+ concentration in the range of 0.05 to 100 μM (Fig. 3). In these studies, concentrated calcium-EGTA buffers including a high total CaCl2 concentration (1 mM) were used to prevent depletion of ionized calcium due to calcium uptake by the microsomes.

The cyclic AMP concentration dependence of microsomal phosphorylation is seen in Fig. 4. Maximum activation of phosphorylation by added protein kinase occurred at about 1 μM added cyclic AMP. Half-maximal activation by cyclic AMP in the presence of protein kinase, based on four independent experiments, was at 0.11 ± 0.02 (S.E.) μM. Slight stimulation of phosphorylation by cyclic AMP seen in the absence of added protein kinase (Fig. 4) probably reflects the presence of a protein kinase in the microsomal preparation. Its sensitivity to cyclic AMP was similar to that catalyzed by the added protein kinase.

The temperature dependence of microsomal phosphorylation was determined under conditions where phosphorylation was linear with respect to both protein kinase concentration (0.05 mg per ml) and incubation time (30 s). An Arrhenius plot (35) of the results obtained when the temperature was varied between 4 and 40° is shown in Fig. 5.

The preceding studies were performed under conditions similar to those used to demonstrate an effect of protein kinase on calcium uptake (22), in which oxalate as well as a high concentration of KCl to decrease the solubility product of calcium oxalate (38) were included in the incubation medium. The effects of 2.5 mM Tris-oxalate and 0.12 mM KCl on microsomal phosphorylation therefore were examined. Oxalate produced less than 6% inhibition of phosphorylation. No consistent effect of high KCl concentrations on microsomal phosphorylation could be demonstrated.

Effect of Epinephrine on Phosphorylation and Adenylate Cyclase—Because cardiac microsomes contain endogenous adenylate cyclase activity (37-39), agents that stimulate cyclic AMP production may be expected to enhance protein kinase-catalyzed microsomal phosphorylation in the absence of added cyclic AMP. Fig. 6 shows stimulation of microsomal phosphorylation after incubation in 10 μM Z-epinephrine in the presence and absence of protein kinase. This stimulation was completely abolished by 20 μM dl-propranolol (Table I). Further evidence that microsomal phosphorylation may be stimulated by exogenously produced cyclic AMP was obtained from measurements of adenylate cyclase activity in cardiac sarcoplasmic reticulum under conditions similar to those described for the phosphorylation studies (see “Experimental Procedure”). dl-Epinephrine (10 μM) produced an approximately 2-fold increase in cyclic AMP when measured after 15 and 30 min of incubation in the presence and absence of protein kinase (Fig. 7). The stimulation of adenylate cyclase by epinephrine was completely abolished by 20 μM dl-propranolol.

Characterization of Acid Precipitable [32P]Phosphate—The acid-precipitable [32P]phosphate measured under standard assay conditions was characterized with respect to its chemical stability...
but significant incorporation of \([a2P]\)phosphate into phospho-microsomes was examined, a significant number of counts was observed at the spot corresponding to phosphothreonine. When microsomes incubated in the presence of cyclic AMP with or without added protein kinase (control microsomes), 86% of the \([32P]\)phosphate was released from phosphorylated microsomes. In the case of microsomes alone, slight increase in calcium uptake rate over a wide range of protein kinase concentrations. A positive correlation is found for both cardiac and skeletal muscle protein kinases paralleled the increase in calcium uptake rate over a wide range of protein kinase concentrations. The present findings indicate that the incorporation of \([32P]\)phosphate from \([\gamma-P]\)ATP into acid-precipitable material ob-

### TABLE I

**Stimulation of microsomal phosphorylation by l-epinephrine**

Cardiac microsomes were incubated for 30 min under standard conditions for measuring phosphorylation in the presence and absence of protein kinase (0.1 mg per ml), 10 \(\mu\)M l-epinephrine, or \(10 \mu\)M l-epinephrine plus 20 \(\mu\)M dl-propranolol. See "Experimental Procedure" for additional details.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Phosphorylation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (\pm S.E.) (N)</td>
<td>(p^a)</td>
</tr>
<tr>
<td></td>
<td>(\text{nmol} P/\text{mg/30 min})</td>
<td></td>
</tr>
<tr>
<td>Without protein kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.78 (\pm 0.08) (4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>l-Epinephrine</td>
<td>1.08 (\pm 0.13) (4)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>l-Epinephrine + dl-propranolol</td>
<td>0.69 (\pm 0.05) (4)</td>
<td></td>
</tr>
<tr>
<td>With protein kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.18 (\pm 0.18) (3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>l-Epinephrine</td>
<td>1.49 (\pm 0.13) (3)</td>
<td></td>
</tr>
<tr>
<td>l-Epinephrine + dl-propranolol</td>
<td>1.28 (\pm 0.20) (3)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

\(a^a\) Based on Student's \(t\) test for paired variates when compared with value on line immediately above.

(Table II). Following incubation in 0.5 \(\times\) NaOH at 90° for 10 min and subsequent washing with trichloroacetic acid, all \([\gamma-P]\) phosphate was released from phosphorylated microsomes. When microsomes were incubated in the absence of cyclic AMP and protein kinase (control microsomes), 86% of the \([\gamma-P]\)phosphate was recovered in the precipitate after treatment with 0.1% trichloroacetic acid for 10 min at 90°. Of the total phosphorylation observed in the presence of cyclic AMP and protein kinase, which was approximately 4 times greater than for the control microsomes (Table II), 89% was recovered in the acid precipitate obtained as described above. Treatment of control microsomes with 0.8 \(\text{m}\) hydroxylamine or 0.8 \(\text{m}\) NaCl resulted in almost complete recovery of the acid-precipitable \([\gamma-P]\)phosphate. Similarly, the recovery in acid precipitates of \([\gamma-P]\)phosphate formed in the presence of cyclic AMP and protein kinase was only slightly reduced after treatment with 0.8 \(\text{m}\) hydroxylamine (Table II).

The distribution of \([\gamma-P]\)phosphate in acid hydrolysates of microsomes incubated in the presence of cyclic AMP with or without added protein kinase is summarized in Table III. When the distribution of \([\gamma-P]\)phosphate in acid precipitates of protein kinase incubated with cyclic AMP but in the absence of microsomes was examined, a significant number of counts was found to be associated with phosphoserine and a lesser number with phosphothreonine. In the case of microsomes alone, slight but significant incorporation of \([\gamma-P]\)phosphate into phosphoserine and phosphothreonine also was seen. When microsomes were incubated with cyclic AMP and the protein kinase, an almost 40-fold increase in counts at the spot on the electrophoretogram corresponding to phosphoserine was observed. A lesser increase in counts corresponding to phosphoserine, approximately 4-fold, was found when microsomes were incubated with cyclic AMP alone. In both cases an increase in counts also was observed at the spot corresponding to phosphothreonine.

**Relationship of Protein Kinase-catalyzed Phosphorylation to Calcium Uptake**—Protein kinase isolated from both bovine heart or rabbit fast skeletal muscle increased the rate of calcium uptake by dog heart sarcoplasmic reticulum, the effects on phosphorylation and calcium uptake of incubating cardiac microsomes with increasing concentrations of both types of protein kinases were determined (Fig. 9). The skeletal muscle protein kinase preparations were less active in causing stimulation of phosphorylation and calcium uptake than were the cardiac protein kinase preparations, but the increasing phosphorylation of cardiac microsomes that was induced by cardiac and skeletal muscle protein kinases paralleled the increase in calcium uptake rate over a wide range of protein kinase concentrations. A positive correlation is found for both cardiac (\(r = 0.96, p < 0.001\)) and skeletal protein kinases (\(r = 0.94, p < 0.001\)) and the best fit lines, calculated by the method of least mean squares with the aid of a PDP-8e computer, were virtually superimposable. The best fit line using combined data is shown in Fig. 10.

**DISCUSSION**

The present findings indicate that the incorporation of \([\gamma-P]\)phosphate from \([\gamma-P]\)ATP into acid-precipitable material ob-

### TABLE II

**Stability of \([pP]\)phosphate formed in presence and absence of cyclic AMP and protein kinase**

Cardiac sarcoplasmic reticulum was incubated under standard conditions (Procedure I) in the presence and absence of 1 \(\mu\)M cyclic AMP and 0.1 mg per ml of bovine cardiac protein kinase. The trichloroacetic acid-precipitable material was subjected to the treatments indicated, each for 10 min, in addition to the standard trichloroacetic acid wash procedure. Percentages indicate the recovery of \([\gamma-P]\)phosphate relative to samples that were maintained on ice in 10% trichloroacetic acid (TCA); the latter were taken as 100%. Further details are described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cyclic AMP + Protein Kinase</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{nmol P/ mg/2 min})</td>
<td>(%)</td>
</tr>
<tr>
<td>None (control = TCA, 90°)</td>
<td>0.264</td>
<td>100</td>
</tr>
<tr>
<td>(\text{NaOH, 0.5 mM at 90°})</td>
<td>0.000</td>
<td>0.088</td>
</tr>
<tr>
<td>TCA, 10%; 90°</td>
<td>0.227</td>
<td>86</td>
</tr>
<tr>
<td>Hydroxylamine, 0.8 mM at 30°</td>
<td>0.260</td>
<td>84</td>
</tr>
<tr>
<td>(\text{NaCl, 0.8 mM at 30°})</td>
<td>0.283</td>
<td>107</td>
</tr>
</tbody>
</table>

The stability of \([\gamma-P]\)phosphate in the presence and absence of cyclic AMP and protein kinase is summarized in Table II. Following incubation in 0.5 \(\times\) NaOH at 90° for 10 min and subsequent washing with trichloroacetic acid, all \([\gamma-P]\) phosphate was released from phosphorylated microsomes. When microsomes were incubated in the absence of cyclic AMP and protein kinase (control microsomes), 86% of the \([\gamma-P]\)phosphate was recovered in the precipitate after treatment with 0.1% trichloroacetic acid for 10 min at 90°. Of the total phosphorylation observed in the presence of cyclic AMP and protein kinase, which was approximately 4 times greater than for the control microsomes (Table II), 89% was recovered in the acid precipitate obtained as described above. Treatment of control microsomes with 0.8 \(\text{m}\) hydroxylamine or 0.8 \(\text{m}\) NaCl resulted in almost complete recovery of the acid-precipitable \([\gamma-P]\)phosphate. Similarly, the recovery in acid precipitates of \([\gamma-P]\)phosphate formed in the presence of cyclic AMP and protein kinase was only slightly reduced after treatment with 0.8 \(\text{m}\) hydroxylamine (Table II).

1-Epinephrine + dl-propranolol...

Z-Epinephrine + dl-propranolol...

PM

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AMP plus 0.1 mg per ml of protein kinase (PK) under the assay prepared from bovine heart (A, 0) or rabbit fast skeletal muscle nor cyclic AMP (X) according to Procedure B described in the text. Dog cardiac microsomes on the concentration of protein kinase fast skeletal muscle protein kinase (O), or neither protein kinase conditions described for this analysis under "Experimental Procedure." Protein kinase was incubated under identical con-

**Fig. 9 (center).** Dependence of microsomal phosphorylation ($\Delta - \Delta$, $\Delta - \Delta$) and calcium uptake (O -- O, O O) by dog cardiac microsomes on the concentration of protein kinase prepared from bovine heart (A, O) or rabbit fast skeletal muscle (A, O).

**Fig. 10 (right).** Relationship of net stimulation of calcium uptake rate by bovine cardiac (O) or rabbit fast skeletal muscle (O) protein kinase to net increase in protein kinase-catalyzed phosphorylation. Data were taken from Fig. 9.

### Table III

<table>
<thead>
<tr>
<th>Conditions</th>
<th>[32P]Phosphate distribution in phosphoserine and phosphothreonine in dog cardiac microsomes and bovine cardiac protein kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[32P]Phosphoserine (A)</td>
</tr>
<tr>
<td>Control...</td>
<td>78 (33)</td>
</tr>
<tr>
<td>PK + cyclic AMP:</td>
<td></td>
</tr>
<tr>
<td>no microsomes...</td>
<td>91 (57)</td>
</tr>
<tr>
<td>Cyclic AMP...</td>
<td>600 (72)</td>
</tr>
<tr>
<td>Cyclic AMP + PK</td>
<td>2860 (79)</td>
</tr>
</tbody>
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半-最大活性化由プロテインキナーゼ触媒活性のマイクロソームphosphorylationは、細胞AMPが存在下で、0.11 ± 0.02（S.E.）μM。同じ値の半最大活性化によって、添加細胞AMPが得られることで、細胞キナーゼ刺激によりカルシウム取込み（22）およびCa2⁺-活性化ATPase（43）。これらの値の類似性は、カルシウムの可逆性を示す細胞質体（40）。表を用いて得られた値が、これらのマイクロソームの準備を示す。

M. Tada, H.-C. Li, and M. A. Kirchberger, manuscript in preparation.

**TABLE III**

[32P]Phosphate distribution in phosphoserine and phosphothreonine in dog cardiac microsomes and bovine cardiac protein kinase

Microsomes (0.5 mg per ml) were incubated for 10 min in the absence (control) and presence of 1 μM cyclic AMP or 1 μM cyclic AMP plus 0.1 mg per ml of protein kinase (PK) under the assay conditions described for this analysis under "Experimental Procedure." Protein kinase was incubated under identical conditions except that microsomes were omitted from the reaction mixture. Protein was hydrolyzed in 6 N HCl and subjected to high voltage paper electrophoresis. Data represent average of duplicate reaction mixtures expressed as counts per 10 min in the region on the electrophoretogram corresponding to either phosphoserine or phosphothreonine. Data obtained from zero time samples have been subtracted from all values. Corrections have been made for breakdown of phosphoserine (31%) and phosphothreonine (8%) during acid hydrolysis. Figures in parentheses represent the percentage of the total counts that migrated from the origin of the chromatogram. Thirty-five per cent of the total counts applied to the chromatogram was found at the origin due to incomplete hydrolysis of protein. The last two columns represent the fold increase in counts relative to control.
and cyclic AMP is, in fact, the result of the membrane phosphorylation that is documented in the present report.

Oscillations were regularly seen during the initial phase of microsomal phosphorylation. For example, after about 10 min of incubation, the rate of microsomal phosphorylation in the presence of cyclic AMP and protein kinase levels off only to increase again and reach a plateau (Fig. 1). This pattern of phosphorylation may be evidence of the presence of two or more interacting enzymes (44), one of which may be a phosphoprotein phosphatase. The oscillations cannot be explained on the basis of a competition between the adenylate cyclase and protein kinase for the substrate ATP because ATP concentrations remain in the millimolar range, well above the apparent $K_m$ of approximately 0.1 mM ATP for microsomal phosphorylation.3

The increase in microsomal phosphorylation seen in the presence of cyclic AMP alone (Fig. 1), which represents increased incorporation of [32P]phosphate into phosphoserine and phosphothreonine (Table III), appears to be catalyzed by an endogenous protein kinase. Similar findings have recently been published by Wray et al. (45) who measured protein kinase activity in cardiac microsomes.

The increase in phosphorylation of cardiac microsomes by protein kinase in the absence of added cyclic AMP (Fig. 1) may reflect the activity of the adenylate cyclase that has previously been found in cardiac microsomal preparations (37-39). Based on data shown in Fig. 7, the concentration of cyclic AMP produced by the microsomal adenylate cyclase is sufficiently high for stimulation of phosphorylation. Alternatively, the observed stimulation of phosphorylation could be the result of activation of protein kinase by substrate protein (46).

Comparison of the extent of microsomal phosphorylation in the absence and presence of high concentrations of KCl, such as are found intracellularly, shows no significant differences. These results may represent additive effects of KCl on several enzymes known to be present in the microsomal preparation. Pronounced effects of KCl on protein kinase activity as well as on phosphatase activity of human lymphocytes have been reported (47).

The Arrhenius plot of data obtained in the study of the effect of temperature on microsomal phosphorylation (Fig. 5) shows a discontinuity at approximately 13°C and is concave downwards. The energy of activation for the microsomal phosphorylation reaction is approximately 10.0 Cal per mole at temperatures ranging from 4 to 13°C and 7.2 Cal per mole at temperatures ranging from 13 to 40°C as calculated from the slopes of the lines drawn by the method of least mean squares. The discontinuity at approximately 13°C may be due to a change in the thermodynamic properties of bovine cardiac protein kinase although definitive conclusions may not be drawn due to the heterogeneity of the membrane preparation.

The possibility that the system of enzymes described above has physiological significance would be supported if naturally occurring agents that increase cyclic AMP also cause stimulation of phosphorylation. The present studies show that 10 $\mu$M epinephrine increases adenylate cyclase activity (Fig. 7) as well as microsomal phosphorylation (Fig. 6) and that this is a $\beta$-adrenergic effect in that this stimulation is completely abolished by dl-propranolol (Fig. 7 and Table I). While the increase in cyclic AMP production in response to epinephrine was approximately 2-fold, the increase in the amount of phosphorylation in response to epinephrine was relatively less. Protein-protein interactions leading to activation of protein kinase such as were mentioned above could be one factor in producing disproportionate increases in cyclic AMP production and microsomal phosphorylation. The relatively small increase in phosphorylation in response to epinephrine in the early part of the time course shown in Fig. 6 could be attributed to a cyclic AMP concentration attained under these conditions (approximately 0.1 $\mu$M) based on data in Fig. 7 which is not sufficient for full stimulation of protein kinase activity.

Evidence presented in this communication, as well as by other investigators, indicates that the sarcoplasmic reticulum contains both an intrinsic protein kinase (45, 48) as well as an adenylate cyclase (37-39). However, protein kinase present in the myoplasm that bathes the sarcoplasmic reticulum and activated by cyclic AMP produced at the plasma membrane may in the intact tissue also play a role in the phosphorylation of the sarcoplasmic reticulum.

Protein kinases derived from different mammalian species, as well as those prepared from different tissues, are capable of stimulating the calcium transport system of sarcoplasmic reticulum isolated from dog heart. Net stimulation of calcium uptake rate paralleled the net increase in protein kinase-catalyzed phosphorylation and this relationship is independent of the source of protein kinase (Fig. 10).

The apparent stoichiometry between maximal protein kinase-induced phosphate incorporation into cardiac microsomes, which is about 1.5 nmoles of P_i per mg of microsomal protein (e.g. Fig. 9), and that reported for the incorporation of phosphate into the acyl phosphate ATPase intermediate (49-51) is approximately 1. Assuming 1 mole of phosphate to be incorporated per mole of a regulatory phosphoprotein whose formation is catalyzed by protein kinase, each mole of this regulatory phosphoprotein would have the ability to influence a single mole of the calcium transport ATPase. Further studies of the proposed regulatory phosphoprotein will be needed to clarify these relationships.

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