Phosphorylation of a 22,000-Dalton Component of the Cardiac Sarcoplasmic Reticulum by Adenosine 3':5'-Monophosphate-dependent Protein Kinase

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SUMMARY

Cardiac microsomes were incubated with [γ-32P]ATP and a cardiac adenosine 3':5'-monophosphate (cyclic AMP)-dependent protein kinase in the presence of ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid. After solubilization in sodium dodecyl sulfate and fractionation by polyacrylamide gel electrophoresis, a single microsomal protein component of approximately 22,000 daltons was found to bind most of the 32P label. The 32P labeling of this component increased several fold when NaF was included in the incubation medium. No other component of cardiac microsomes, including sarcoplasmic reticulum ATPase protein, contained significant amounts of 32P label. This 22,000-dalton phosphoprotein formed by cyclic AMP-dependent protein kinase had stability characteristics of a phosphoester rather than an acyl phosphate. Washing of microsomes with buffered KCl did not decrease the amount of 32P labeling to the 22,000-dalton protein, suggesting that this protein is associated with the membranes of sarcoplasmic reticulum rather than being a contaminant from other soluble proteins. The 22,000-dalton protein was susceptible to trypsin. Brief digestion with trypsin in the presence of 1 M sucrose did not significantly affect microsomal calcium transport activity, but prevented both subsequent phosphorylation of the 22,000-dalton protein and stimulation of calcium uptake by cyclic AMP-dependent protein kinase, suggesting that this protein is a modulator of the calcium pump.

These results are consistent with previous findings (KIRCHBERGER, M. A., TADA, M., and KATZ, A. M. (1974) J. Biol. Chem. 249, 6166-6173; TADA, M., KIRCHBERGER, M. A., REPKE, D. I., AND KATZ, A. M. (1974) J. Biol. Chem. 249, 6174-6180) that cyclic AMP-dependent protein kinase-catalyzed phosphorylation is associated with stimulation of calcium transport in the cardiac sarcoplasmic reticulum, and further indicate that this phosphorylation occurs at a component of low mass (22,000 daltons) of the cardiac sarcoplasmic reticulum which, while separable from the calcium transport ATPase protein (100,000 daltons) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, has the ability to regulate calcium transport by the cardiac sarcoplasmic reticulum.

In previous communications we have reported that a cardiac cyclic adenosine 3':5'-monophosphate-dependent protein kinase enhances both calcium uptake and Ca2+-activated ATPase activity of a dog cardiac microsomal preparation (4, 5) which consists mainly of fragmented sarcoplasmic reticulum (6). These functional alterations in calcium transport are correlated with phosphorylation of microsomes by cyclic AMP-dependent protein kinases (7). The microsomal phosphoprotein has stability characteristics of a phosphoester in which the phosphate is incorporated largely into serine (7). Its formation does not require Ca2+ (7). Cardiac microsomes can form another type of phosphoprotein, an intermediate of calcium transport ATPase protein (100,000 daltons) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a single microsomal protein component of approximately 22,000 daltons was found to bind most of the 32P label. The 32P labeling of this component increased several fold when NaF was included in the incubation medium. No other component of cardiac microsomes, including sarcoplasmic reticulum ATPase protein, contained significant amounts of 32P label. This 22,000-dalton phosphoprotein formed by cyclic AMP-dependent protein kinase had stability characteristics of a phosphoester rather than an acyl phosphate. Washing of microsomes with buffered KCl did not decrease the amount of 32P labeling to the 22,000-dalton protein, suggesting that this protein is associated with the membranes of sarcoplasmic reticulum rather than being a contaminant from other soluble proteins. The 22,000-dalton protein was susceptible to trypsin. Brief digestion with trypsin in the presence of 1 M sucrose did not significantly affect microsomal calcium transport activity, but prevented both subsequent phosphorylation of the 22,000-dalton protein and stimulation of calcium uptake by cyclic AMP-dependent protein kinase, suggesting that this protein is a modulator of the calcium pump.

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

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2640
mately 22,000 daltons, which can be separated from ATPase protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This 22,000-dalton protein appears to have the ability to modulate calcium transport by the cardiac sarcoplasmic reticulum.

**EXPERIMENTAL PROCEDURE**

**Materials**

Cardiac microsomes were prepared from dog heart ventricle according to the procedures of Harigaya and Schwartz (8) with minor modifications (7). Cyclic AMP-dependent protein kinase was purified through the DEAE-cellulose chromatography step from bovine hearts, according to the method of Miyamoto et al. (14). Cyclic AMP-dependent protein kinase was purified through the DEAE-cellulose chromatography step from bovine hearts, according to the method of Miyamoto et al. (14).

**Methods**

Phosphorylation of Microsomes by Cyclic AMP-dependent Protein Kinase

**Protein Kinase**

Microsomal protein was phosphorylated at 25° for 10 min in one of the following reaction mixtures in a total volume of 0.2 ml unless otherwise stated.

**Reaction Mixture A**—Mixture A consisted of 40 mM histidine buffer (pH 6.8), 0.12 mM MgCl₂, 5 mM MgSO₄, 5 mM [γ-³²P]ATP (2 cpm per µl), 10 mM EGTA, 5 mM NaN₃, and 1 to 3 mg per ml of microsomal protein with or without 25 mM NaF and/or 1 µM cyclic AMP and various concentrations of protein kinase.

**Reaction Mixture B**—Mixture B consisted of 40 mM histidine buffer (pH 6.8), 0.12 mM KCl, 5 mM MgCl₂, 5 mM [γ-³²P]ATP (2 cpm per µl), and 0.5 mg per ml of microsomal protein with or without 1 µM cyclic AMP and various concentrations of protein kinase.

**Reaction Mixture C**—Mixture C was identical with Reaction Mixture B except that unlabelled ATP was used. Reactions were started by the addition of microsomes. Reactions A and B were terminated by the appropriate methods as described below, and phosphorylated microsomes were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Microsomes incubated in Reaction Mixture C were subjected to assay for ATPase and calcium uptake as described below.

**Determination of Phosphoprotein by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**

Reactions A and B were terminated and microsomal protein was solubilized by the procedures described by Pucell and Martonosi (17) modified as described below.

**a. Termination with Sodium Dodecyl Sulfate (Procedure 1)**—To the reaction mixture (0.2 ml) was added 0.1 ml of a solution containing sodium dodecyl sulfate, EDTA, and β-mercaptoethanol, to give final concentrations of 0.1%, 0.1 mM, and 1%, respectively. After standing several minutes on ice, this mixture was incubated for approximately 10 min at 37° to solubilize the microsomal protein and a 0.3-ml aliquot was added to the 0.2 ml of a solution containing 20 mM sodium phosphate buffer (pH 7.2), 1.0 mM EDTA, 1% β-mercaptoethanol, 50% glycerol, and 0.005% bromphenol blue. An aliquot (90 to 100 µl) of this solution, containing 40 to 150 µg of microsomal protein, was applied to the gel for electrophoresis.

**b. Termination with Trichloroacetic Acid (Procedure 2)**—The reaction was terminated by the addition of 2 ml of ice-cold 10% trichloroacetic acid containing 0.1 mM KH₂PO₄. After centrifugation at 1500 X g for 5 min at 4°, the precipitated protein was suspended in 2 ml of cold distilled water and centrifuged again. The precipitate was applied to 5 ml of a solution containing 20% sodium dodecyl sulfate, 0.1 mM EDTA, 1% β-mercaptoethanol, and 20 mM sodium phosphate buffer (pH 7.2). This mixture was incubated at 37° for approximately 10 min to solubilize the microsomal protein, after which 0.1 ml of a solution which contained 20 mM sodium phosphate buffer (pH 7.2), 0.1 mM EDTA, 1% β-mercaptoethanol, 50% glycerol, and 0.005% bromphenol blue were added. This mixture, which contained 0.1 to 0.3 µg of microsomal protein, was applied to the gel for electrophoresis.

**Procedures for sodium dodecyl sulfate-polyacrylamide gel electrophoresis** were essentially those of Weber and Osborn (18). After electrophoresis, duplicate gels were analyzed for the distribution of protein radioactivity. For determination of protein radioactivity, the gels were sliced at intervals of 1 mm with a gel slicer immediately after electrophoresis. Each slice was counted in 5 ml of toluene-base scintillation fluid (4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis[2-(4-methyl-5-phenylxazoyl)]benzene in 1 liter of toluene) by liquid scintillation spectrometry. Digestion of sliced gels with H₂O₂ did not increase significantly the efficiency of counting. Mobilities of protein bands and radioactive peaks were determined as retardation factor, Rs, relative to the position of the tracking dye. For determination of molecular weight, albumin (bovine serum), ovalbumin, chymotrypsinogen A (bovine pancreas), myoglobin (sperm whale), and cytochrome c (horse heart) were used as markers.

**Stability of Phosphoprotein Formed by Protein Kinase**

Microsomes were phosphorylated in Reaction Mixture A, described above, and the reaction was terminated with 2 ml of 10% trichloroacetic acid containing 0.1 mM KH₂PO₄. The samples were centrifuged at 1500 X g for 5 min, and the pellet was treated under several conditions as described below, after which it was solubilized and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

a. **Treatment with Acid and Alkali**—The pellet was incubated in 0.2 ml of 0.5 N NaOH at 0, 37, and 90°, and in 0.2 ml of 10% trichloroacetic acid at 90°. For a control, the pellet was left at 0° in 0.2 ml of 10% trichloroacetic acid after electrophoresis. Each slice was counted in 5 ml of toluene-base scintillation fluid (4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis[2-(4-methyl-5-phenylxazoyl)]benzene in 1 liter of toluene) by liquid scintillation spectrometry. Digestion of sliced gels with H₂O₂ did not increase significantly the efficiency of counting. Mobilities of protein bands and radioactive peaks were determined as retardation factor, Rs, relative to the position of the tracking dye. For determination of molecular weight, albumin (bovine serum), ovalbumin, chymotrypsinogen A (bovine pancreas), myoglobin (sperm whale), and cytochrome c (horse heart) were used as markers.

**Treatment of Microsomes with Tryptsin**

Microsomes (3 to 6 mg per ml) were digested with trypsin at 25° in 40 mM histidine buffer (pH 6.8), 0.12 M KCl, and various concentrations of trypsin in the presence and absence of 1 M sucrose. The weight ratio of trypsin to microsomes was 1:20 in most experiments. Reactions were started by the addition of trypsin. Aliquots...
taken at various time intervals were added to tubes containing trypsin inhibitor that gave a trypsin to trypsin inhibitor ratio of 1:2 by weight. For zero time, a mixture of trypsin and trypsin inhibitor was added. Phosphoprotein formation in trypsin-treated microsomes was determined by incubation at Reaction Mixture A or B and electrophoresis on sodium dodecyl sulfate-polyacrylamide gels as described above. Calcium uptake and Ca²⁺-activated ATPase activity of trypsin-treated microsomes were measured by the procedures described previously (5).

In the experiments in which phosphorylated microsomes were treated with trypsin, microsomes were phosphorylated in Reaction Mixture A or C for 10 min at 25°C, after which trypsin was added at a microsomal protein to trypsin ratio of 20:1. In some experiments, phosphorylated microsomes were washed with buffer solution before treatment with trypsin. At time intervals after the addition of trypsin, trypsin inhibitor was added (trypsin to trypsin inhibitor = 1:2). The amount of phosphoprotein formed in these microsomes (Reaction Mixture A) was determined by gel electrophoresis as described above, and calcium uptake and Ca²⁺-activated ATPase activity (Reaction Mixture C) were measured as described previously (5).

Determination of RNA

Cardiac microsomes were digested in KOH and RNA content was determined spectrophotometrically by the method of Fleck and Munro (20).

RESULTS

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Microsomal Proteins—Canine cardiac microsomes that were solubilized in sodium dodecyl sulfate showed several distinct protein bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A major protein band, a, five secondary bands, b to f, and a diffuse band, z, were found when small amounts (42 μg) of microsomal protein were applied to the gel (A, Fig. 1). Washing of the microsomes with 0.6 M KCl caused no obvious change in this pattern, and no low molecular weight components were found in the supernatant after KCl washing. When larger amounts of cardiac microsomal protein were applied to the gels, less distinct tertiary bands, including Bands w, x, and y, became visible (Fig. 2B). When the periodic acid-Schiff method was applied, only Band z was stained. Bovine cardiac protein kinase also produced several protein bands when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (B, Fig. 1). Electrophoresis of a mixture of cardiac microsomal protein and

![Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein markers (A) and canine cardiac microsomes labeled with ³²P in the presence of bovine cardiac protein kinase and cyclic AMP (B and C). The top of the gel is at the left; proteins migrate toward the anode (at right). The right margin of each figure corresponds to the forefront of the tracking dye. A, protein markers are: BSA; bovine serum albumin; OVA, ovalbumin; CHY, chymotrypsinogen A; MYO, myoglobin; CYT, cytochrome c. The proteins were incubated for 2 hours at 37°C in 1% sodium dodecyl sulfate, 1% β-mercaptoethanol, and 10 mM sodium phosphate buffer (pH 7.2), and aliquots (0.02 ml) of the solutions containing 3 μg of each protein marker were applied to the gels. Electrophoresis was performed as described in the text. Each point represents an average of four determinations. B and C, microsomes (1.6 mg per ml) were phosphorylated in Reaction Mixture A (see “Methods”) with 0.8 mg per ml of bovine cardiac protein kinase and 1 μM cyclic AMP in the presence (●) and absence (○) of 25 mM NaF. Reactions were terminated by Procedure I, and electrophoresis was carried out, as described in the text, with application of 0.112 mg of microsomal protein + 0.056 mg of protein kinase for determining protein distributions (B), and 0.056 mg of microsomal protein + 0.028 mg of protein kinase for determining the distributions of radioactivity (C). In B, only the electrophoretogram of microsomal protein incubated in the presence of 25 mM NaF is shown since omission of NaF resulted in similar protein distributions.

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoreograms of canine cardiac microsomes (A) and bovine cardiac protein kinase (B). Microsomal protein (1.2 mg per ml) and protein kinase (1.4 mg per ml) in 50 mM histidine buffer (pH 6.8) and 0.005 M KCl in a total volume of 0.2 ml were solubilized in sodium dodecyl sulfate (Procedure I, see “Methods”), and aliquots (0.1 ml) of solution containing 42 μg of microsomal protein and 50 μg of protein kinase were applied to Gels a and b, respectively. Electrophoresis was carried out as described in the text. Proteins were applied at the top of the gels and migrated toward the anode at the bottom.
protein kinase gave the pattern expected from the combination of both proteins (Fig. 2B).

**Phosphorylation of 22,000-dalton Component by Protein Kinase**

Cardiac microsomes phosphorylated by cyclic AMP and protein kinase showed a single significant peak of radioactivity (Peak II, Fig. 2C) when $\gamma$P labeling was determined by measurement of the radioactivity of sliced gels. When protein kinase and cyclic AMP were omitted from the incubation medium, no clear peaks were seen at this area or any other area of the gel. A peak of much higher radioactivity was found at this same location (Peak II, Fig. 2C) when 25 mM NaF was included in the reaction medium in order to inhibit phosphoprotein phosphatase activity present in microsomal preparations.\(^7\) The increase of $\gamma$P labeling due to NaF was approximately 5-fold when the amounts of $\gamma$P were estimated from the area of Peak II. In addition to the major peak, two peaks of much less radioactivity were noted in the presence of NaF (Peaks I and III, Fig. 2C). Peak I may represent $\gamma$P labeling of one of the subunits of protein kinase since $\gamma$P labeling of the same extent at the same location as Peak I was found when protein kinase was incubated in the absence of cardiac microsomes under identical conditions. This peak of radioactivity corresponded to the protein Band 3 of approximately 55,000 daltons seen in Fig. 1B.

The major peak of radioactivity (Peak II, Fig. 2C) corresponded to one of the minor components (w, Fig. 2B) of microsomes. Among 12 determinations, using 5 different microsomal preparations, Peak II was seen at $R_F 0.62 \pm 0.02$ (S.D.), whereas the minor protein band w was found at $R_F 0.63 \pm 0.01$ (S.D.). Based on the calibration curve (Fig. 2A), the apparent molecular weight of this phosphoprotein component was estimated from the latter $R_F$ value to be 22,000 $\pm$ 1,000. An additional minor peak of radioactivity (Peak III, Fig. 2C) was seen when microsomes were phosphorylated in the presence of NaF. The distribution of radioactivity was similar when the specific activity of the [$\gamma$P]ATP was increased 100-fold. When the total $\gamma$P-phosphate found in Peak II in the presence of NaF (0.035 nmol) is compared with the total microsomal protein applied (0.056 mg) in Fig. 2C, approximately 0.63 nmol of phosphate were found to be incorporated per mg of microsomal protein. This value varied among a number of microsomal preparations within the range of 0.5 to 1.0 nmol of phosphate per mg of microsomal protein, which is in good agreement with the value obtained under similar conditions by the trichloroacetic acid precipitation procedure (7).

**Stability of Phosphoprotein**—In order to study the nature of phosphate-binding to the 22,000-dalton component, microsomes phosphorylated in the presence of protein kinase and [$\gamma$P]ATP were treated under various conditions, after which they were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the amounts of $\gamma$P bound to this component were estimated by trichloroacetic acid precipitation (7).

### Table I

| Treatment | $\gamma$P bound to 22,000-dalton component (%) |
|-----------|---------------------------------------------|
| Control (10% trichloroacetic acid, 0°) | 100 |
| 0.5 N NaOH, 0° | 87 |
| 0.5 N NaOH, 37° | 30 |
| 0.5 N NaOH, 90° | <1 |
| Control (0.8 M NaCl) | 100 |
| 0.8 M hydroxylamine | 96 |
| Control (10% trichloroacetic acid, 0°) | 100 |
| Chloroform-methanol, 0° | 99 |
| Acetone, 0° | 88 |

The relationship between protein kinase-induced stimulation...
of microsomal calcium transport and protein kinase-catalyzed phosphorylation of the 22,000-dalton protein was investigated by determining whether protein kinase and cyclic AMP could stimulate calcium uptake by microsomes which, after 20 min of trypsin treatment in 1 M sucrose, have retained calcium uptake activity but lost most of the ability to be phosphorylated by protein kinase (Fig. 6). In contrast to control microsomes, where the rate of calcium uptake was greatly stimulated by treatment with protein kinase and cyclic AMP, calcium uptake by microsomes that had been treated with trypsin was not stimulated after incubation with protein kinase and cyclic AMP.

**Trypsin Treatment of Phosphorylated Microsomes**—The 22,000-dalton component could be shown to be resistant to trypsin after it had been phosphorylated by protein kinase prior to the addition of trypsin. Microsomes were phosphorylated under standard conditions described previously (5) with 1 mM CaCl\(_2\) (Ca-EGTA buffer containing 125 mM CaCl\(_2\)) and 37 \(\mu\)g per ml of microsomal protein. TRYPSINIZED microsomes were incubated with trypsin (0.1 mg per ml) for 20 min under standard conditions in the presence of 1.0 M sucrose. Aliquots of control (○) and trypsin-treated (△) microsomes were incubated with protein kinase (PK) and 1 mM cyclic AMP (cAMP) in Reaction Mixture C. Aliquots of these mixtures were subsequently incubated in calcium uptake assay media containing 1 mM Ca\(^{2+}\); the final concentration of microsomal protein was 25 \(\mu\)g per ml.

**FIG. 6.** Lack of protein kinase effect on calcium uptake by trypsin-treated cardiac microsomes. Microsomes (1.08 mg per ml) were incubated with and without 0.1 mg per ml of trypsin for 20 min under standard conditions in the presence of 1.0 M sucrose. Aliquots of control (○) and trypsin-treated (△) microsomes were incubated with (closed symbols) and without (open symbols) 0.5 mg per ml of protein kinase (PK) and 1 mM cyclic AMP (cAMP) in Reaction Mixture C. Aliquots of these mixtures were subsequently incubated in calcium uptake assay media containing 1 mM Ca\(^{2+}\); the final concentration of microsomal protein was 25 \(\mu\)g per ml.
kinase induce marked stimulation of calcium uptake and Ca\(^{2+}\). Currently, a phosphoester phosphoprotein is formed in these membranes which is chemically different from the phosphoprotein intermediate of the calcium transport ATPase (7). These findings suggest that the protein kinase catalyzes the formation of a phosphoprotein which stimulates the calcium pump of the cardiac sarcoplasmic reticulum. The present study demonstrates that the protein kinase catalyzes phosphorylation of a 22,000-dalton protein which is electrophoretically distinct from the calcium transport ATPase protein of approximately 100,000 daltons and provides support for the view that this 22,000-dalton phosphoprotein has a regulatory role in calcium transport by cardiac sarcoplasmic reticulum.

Cardiac microsomes that have been solubilized in sodium dodecyl sulfate can be fractionated into several components by polyacrylamide gel electrophoresis (Fig. 1A). The major component, which can undergo phosphorylation to form acyl phosphoprotein, an intermediate of ATPase, in that its mobility is similar to that of the previously described ATPase of cardiac (10) and skeletal (17, 24) sarcoplasmic reticulum. Band a represents phospholamban (25). Some of the other bands of cardiac microsomes shown in Fig. 1 may be analogous to the calcium-binding proteins of skeletal sarcoplasmic reticulum (25, 26). However, none of these previously described components served as a substrate for protein kinase-catalyzed phosphorylation (Fig. 2).

**DISCUSSION**

Cyclic AMP and cardiac cyclic AMP-dependent protein kinase induce marked stimulation of calcium uptake and Ca\(^{2+}\). Activated ATPase activity of cardiac microsomes (5, 7). Concurrently, a phosphoester phosphoprotein is formed in these membranes which is chemically different from the phosphoprotein intermediate of the calcium transport ATPase (7, 22, 23). These findings suggest that the protein kinase catalyzes the formation of a phosphoprotein which stimulates the calcium pump of the cardiac sarcoplasmic reticulum. The present study demonstrates that the protein kinase catalyzes phosphorylation of a 22,000-dalton protein which is electrophoretically distinct from the calcium transport ATPase protein of approximately 100,000 daltons and provides support for the view that this 22,000-dalton phosphoprotein has a regulatory role in calcium transport by cardiac sarcoplasmic reticulum.

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The phosphoprotein formed in the presence of protein kinase differs from the phosphoprotein intermediate of the calcium transport ATPase in several aspects. Unlike the ATPase intermediate, this phosphoprotein has the stability characteristics of a phosphoester (Table I) and its formation does not require Ca\(^{2+}\) (7). The possibility that it is primarily phospholipid can be excluded because of its trypsin sensitivity and because neither chloroform-methanol nor acetone extracted the phosphate (Table I). The 22,000-dalton component was not stained by the periodic acid-Schiff method, indicating that it is not a glycoprotein.

Two peaks of low radioactivity were seen in addition to the main peak of radioactivity that is associated with the 22,000-dalton phosphoprotein (Fig. 2C). Peak 1, which was seen when protein kinase was incubated in the absence of microsomes, was virtually unaffected by subsequent treatment with trypsin. However, none of these previously described components served as a substrate for protein kinase-catalyzed phosphorylation (Table II).

### Table II

**Effect of trypsin treatment on calcium uptake by phosphorylated microsomes**

Microsomes (1.0 mg per ml) were phosphorylated under standard conditions (Reaction Mixture C) with 1.0 mg per ml of protein kinase and 1 μM cyclic AMP and ATP in a total volume of 1.4 ml. At 9 min after start of the phosphorylation, 1.2 ml of 2.5 mM sucrose (pH 6.8) were added to give final sucrose concentration of 1.0 M. Trypsin (90 μg in 20 μl) was added 1 min after the addition of sucrose. At indicated times after the addition of trypsin, 0.5-ml aliquots were taken and added to 50 μl of solution containing 30 μg of trypsin inhibitor. Of each of the resulting mixtures, 0.4 ml was incubated in 4 ml of calcium uptake assay media containing 1 μM Ca\(^{2+}\); the final concentration of microsomal protein was 54 μg per ml. The rate of calcium uptake of control microsomes, that were incubated in the absence of protein kinase and cyclic AMP, was 0.099 amol of calcium taken up per min per mg of protein under conditions described above.

| Digestion time | Rate of calcium uptake (µmol Ca/min/mg protein) |
|---------------|---------------------------------------------|
| 0             | 0.130                                       |
| 5             | 0.128                                       |
| 10            | 0.120                                       |
| 20            | 0.118                                       |
| 40            | 0.114                                       |

*We have tentatively named this phosphoprotein "phospholamban" (1-3).
may represent the phosphorylation of one component of the protein kinase preparation (Band 3 in Fig. 1B) whose molecular weight is approximately 50,000 to 60,000. This finding is consistent with the results of Erlichman et al. (27) who reported autophosphorylation of the cyclic AMP-binding subunit of bovine cardiac protein kinase (55,000 daltons). Peak III which was seen only in the presence of NaF may correspond to one or more components of low molecular weight. However, the low radioactivity and the diffuse nature of this peak precluded further analysis.

The 22,000-dalton component of cardiac sarcoplasmic reticulum appears to be similar to a protein of less than 30,000 daltons reported by Andrew et al. (28) in rat skeletal muscle microsomes. The recent observation that a ribosomal protein of approximately 27,000 daltons is phosphorylated in the reticulocyte by a cyclic AMP-dependent protein kinase (30) raises the possibility that the 22,000-dalton phosphoprotein of cardiac microsomes, which is present in low quantities, represents a contaminant derived from ribosomes. This seems unlikely, however, because the average content of ribosomal protein, estimated from RNA content, is less than 0.4% of the total microsomal protein, whereas the minimal content of the 22,000-dalton phosphoprotein in cardiac microsomes is 4%, if one assumes each mole of the latter to incorporate a single mole of phosphate. The possibility that the 22,000-dalton component might be a polyvalent phosphoprotein of ribosomal origin can also be excluded by the finding that the 32P-labeled phosphoprotein, after treatment with LiCl and urea (31, 32) did not migrate to the cathode in polyacrylamide gels that contained 8 M urea at pH 4.5, in contrast to the behavior of known ribosomal proteins. Furthermore, a single major peak of radioactivity in phosphorylated cardiac microsomes would not be expected if the phosphoprotein were derived from a ribosomal contaminant because mammalian ribosomes incorporate the γ-32P of ATP into a number of phosphoproteins when phosphorylation is carried out in vitro (33-36). This is in contrast to the single phosphoprotein band seen when phosphorylation is carried out in undisrupted cells (30, 34). The present findings, in which phosphorylation of a disrupted cell fraction was examined, do not, therefore, indicate that the 22,000-dalton phosphoprotein is part of a ribosomal contaminant.

The phosphoprotein of cardiac sarcoplasmic reticulum has a molecular weight similar to that of troponin I (37), the component of troponin which inhibits actomyosin ATPase. Troponin I from skeletal muscle has been reported to be phosphorylated by phosphorylase kinase (38) and canine cardiac troponin was recently reported to be phosphorylated by protein kinase (39). It is unlikely, however, that troponin I, which might be introduced as a contaminant from the contractile proteins, accounts for the phosphorylation documented in the present report because phosphorylation of cardiac microsomes did not decrease after washing with 0.6 M KCl, a step that reduces contamination from the contractile proteins. The present results thus suggest that the phosphoprotein is associated with the membranes of the sarcoplasmic reticulum rather than being a contaminant from either ribosomal or soluble proteins. The trypsin sensitivity of the phosphoprotein is greater than that of calcium transport system (Fig. 5), suggesting that the phosphoprotein may be related to the outer surface of the membrane of the sarcoplasmic reticulum.

The present study indicates that the previously suggested regulatory function of protein kinase-catalyzed phosphoprotein formation in the stimulation of cardiac transport by calcium sarcoplasmic reticulum (4, 5, 7) is mediated by phosphorylation of a 22,000-dalton protein. In microsomes treated with trypsin under conditions where calcium transport activity is preserved but in which the 22,000-dalton protein loses its ability to be phosphorylated, calcium uptake could not be stimulated by incubation with cyclic AMP and protein kinase (Fig. 6). Furthermore, in phosphorylated microsomes, whose 22,000-dalton component is resistant to subsequent trypsin treatment (Fig. 7), the stimulated calcium uptake is not lost following exposure to trypsin (Table II). Regulation of calcium transport by phosphorylation of this 22,000-dalton protein appears to be reasonable from a stoichiometric standpoint as similar amounts of phosphate are incorporated into this protein and into the acyl phosphoprotein intermediate of the calcium transport ATPase (7). Further studies on the interaction between purified 22,000-dalton phosphoprotein and the calcium pump will be necessary to define their precise relationship.

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