Cyclic Adenosine Monophosphate Dependent and Independent Phosphorylation of Sarcolemma Membrane Proteins in Perfused Rat Heart†

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ABSTRACT: This study was initiated in order to elaborate further on the mechanism by which epinephrine modulates cardiac function via protein phosphorylation. A membrane fraction has been isolated from freeze-clamped perfused rat heart that contains two phosphoproteins. These proteins have molecular weights of 36000 (A protein) and 27000 (B protein). The phosphorylation of the A protein occurs during the equilibration of the heart with inorganic [^{32}P]phosphate. The phosphorylation of the B protein occurs in response to epinephrine. The A and B proteins are apparently identical with two phosphoproteins in enriched preparations of sarcolemma. The protein of the sarcolemma preparation equivalent to the A protein is phosphorylated in vitro by both cAMP-independent and cAMP-dependent protein kinases. The phosphorylation of the protein of the sarcolemma preparation equivalent to the B protein is catalyzed by the cAMP-dependent protein kinase. Thus the patterns of phosphorylation of these proteins in vivo and in vitro are compatible. The phosphorylation of the B protein has been documented in vitro to modulate calcium transport (Will, H., et al. (1973) *Acta Biol. Med. Ger. 31*, 45–52), but the response to epinephrine in the perfused heart is not apparently coordinated with the catecholamine-induced inotropic effect.

The regulation of cardiac contraction by β-adrenergic stimulation is an event that has been shown to fulfill the four criteria defined by Sutherland to evaluate whether cAMP is the mediator of a hormonal action. In a wide range of mammalian tissues, the regulation of protein phosphorylation by cAMP has been implicated as a major mechanism of action of the cyclic nucleotide. In cardiac muscle the three prime candidates as regulatory phosphoproteins for the control of contraction are troponin (England, 1975, 1976; Stull & Buss, 1977), sarcoplasmic reticulum membrane protein (phospholamban) (Kirchberger et al., 1974; Tada et al., 1974; La Raia & Morkin, 1974; Nayler & Berry, 1975), and sarcolemma membrane protein (Sulakhe et al., 1976; Hui et al., 1976; Krause et al., 1975). Of these only the phosphorylation of troponin has so far been amenable to investigation at the level of the intact cell. England (1975, 1976) has shown that a correlation exists between the incorporation of phosphate into the troponin I subunit and the onset of increased contractile force in perfused rat hearts stimulated by either epinephrine or isoproterenol. This phosphorylation is catalyzed by the cAMP-dependent protein kinase (England, 1977) and has been proposed to be associated with an increased requirement for calcium for the activation of actomyosin ATPase (Ray & England, 1976). England (1976), however, also showed by using pulse perfusions with isoproterenol that, when contractile force was decreasing after previous stimulation, there was no corresponding fall in troponin I phosphorylation. In addition, glucagon-stimulated increase in cardiac contractile force precedes a significant increase in phosphorylation of troponin I. Thus the conclusion has been reached (England, 1976) that, although the phosphorylation of troponin I has a primary role in the modulation of contraction, troponin I phosphorylation is not always associated with increased contractile force. Apparently, therefore, the regulation of contraction by cAMP involves more than the phosphorylation of a single protein. Kirchberger et al. (1974, 1976) and Tada et al. (1974, 1975) have shown that the cAMP-dependent protein kinase catalyzes the phosphorylation of a 22000-dalton protein of cardiac vesicles enriched in sarcoplasmic reticulum, designated phospholamban, and that a correlation exists between the degree of phosphorylation of phospholamban and an enhancement of calcium transport and/or uptake. Thus it has been proposed that the phosphorylation of sarcoplasmic reticulum could, by modulating Ca^{2+} concentrations, mediate some component of the cardiac contractile response initiated by cAMP. A third potential site for the regulation of contraction is the sarcolemma which in cardiac muscle has a primary role in the control of intracellular Ca^{2+} concentration (for a review, see Langer, 1973). Will et al. (1973), Sulakhe et al. (1976), and Hui et al. (1976) have each indicated that cAMP-dependent protein kinase stimulates calcium binding and/or calcium transport of isolated sarcolemma membranes. For neither sarcoplasmic reticulum nor sarcolemma has the phosphorylation state of the membrane proteins been determined for the intact cardiac cell. Thus, despite the in vitro studies, it has been difficult to assess the role of the regulation of Ca^{2+} transport in the mediation of enhanced contraction that occurs in response to cAMP. The primary technical difficulty which impedes this evaluation is that the established isolation procedures for sarcolemma or sarcoplasmic reticulum cannot be used with tissue obtained by freeze-clamping; yet it is essential to use the latter procedure if correlations are to be made between the phosphorylation of membrane proteins and the rapid onset of contraction in response to a cAMP signal. In addition, as has been well established for the phosphorylase system, it is essential to inhibit both dephosphorylation and phosphorylation reactions during tissue extraction. Normally this can be achieved by the addition of EDTA and NaF but such additions further compromise the isolation of sarcolemma and sarcoplasmic reticulum by the established procedures.

In this presentation we report the isolation of a membrane fragment from freeze-clamped perfused rat heart that contains two phosphoproteins; evidence is presented that these are components of the sarcolemma. The phosphorylation of one of these proteins would appear to be primarily catalyzed by

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a cAMP-independent protein kinase. The phosphorylation of the second is cAMP-dependent and occurs in response to epinephrine.

Materials and Methods

Heart Perfusions. Hearts from male Sprague-Dawley rats (250–350 g) were perfused essentially as described by England (1975, 1976). Briefly, hearts were removed under Nembutal-induced anesthesia and perfused by the Langendorff technique with Krebs–Henseleit bicarbonate buffered medium (Krebs & Henseleit, 1932) containing 11 mM glucose but with the P_i concentration reduced to 0.12 mM. The latter permits a higher intracellular specific activity of [γ-^{32}P]ATP to be achieved. The characteristics of P_i uptake into the cardiac cell and the formation of [γ-^{32}P]ATP have been described previously (England & Walsh, 1976). The hearts were perfused by drip-through with nonradioactive medium for an initial 5-min period and then perfused for 30 min with 17.5 mL of recirculating medium containing 0.2–0.35 mCi of $^{32}P_i$/mL. At the termination of this equilibration period, the specific activity of [γ-^{32}P]ATP, determined as described previously (England & Walsh, 1976), was between 60 and 150 dpm per pmol. Following the recycling perfusion with $^{32}P_i$, the hearts were either stimulated with the indicated concentration of epinephrine (drip-through perfusion) or perfused with control medium for an equivalent time period. Contractile force was measured at approximately 80% of the length tension curve with a force-displacement transducer (E and M Instrument Co.) attached by 000 silk thread to the apex of the heart. Flow of perfusion media was regulated by all-Teflon solenoids (Angar Scientific Corp.) which also activated the physiograph for determinations of the interval of catecholamine stimulation. Perfusion temperature was 37 °C. At the termination of the perfusion, hearts were freeze-clamped by the method of Wollenberger et al. (1960) and powdered in a precooled percussion mortar. The specific activities of [γ-^{32}P]ATP in control or epinephrine stimulated tissue were not statistically significantly different.

Preparation of Cardiac Membrane Fractions. Three membrane fractions were used for these studies.

(1) Membranes from Freeze-Clamped Perfused Hearts (Designated the 50K Fraction). This fraction was prepared from hearts perfused as described above, with inorganic [^{32}P]phosphate or with nonradioactive media under equivalent conditions (as indicated for each experiment). The frozen powdered tissue (~1 g) was simultaneously thawed and homogenized in 10 mL of 30 mM Tris-Cl, pH 7.4, containing 5 mM EDTA, 30 mM potassium chloride, 100 mM sodium fluoride, and 1 mM phenylmethanesulfonyl fluoride (previously dissolved in 0.01 volume of ethanol) in a Potter-Elvehjem Teflon/glass homogenizer. The homogenate was centrifuged at 20000*g* for 15 min. To the supernatant, obtained following filtration through glass wool, was added 100 μg of bacterial amylase (87 units); the solution was incubated at 0 °C for 30 min and subsequently centrifuged at 39000*g* for 15 min. To the resultant supernatant was added 2 mL of goat anti-rabbit phosphorylase kinase serum;[1] the solution was incubated at 30 °C for 30 min, on ice for 90 min, and subsequently centrifuged at 10000*g* for 15 min. Ten milliliters of the resultant solution was layered on top of 16.6 mL of 20 mM Tris-Cl, pH 7.4, containing 250 mM sucrose, 100 mM potassium chloride, 0.1 mM EDTA, and 100 mM sodium fluoride and centrifuged in a Beckman Ti 60 rotor at 50000 rpm for 6 h at 4 °C. The supernatant solution was discarded and the pellet stored frozen at −20 °C for 12 h. For samples for $NaDodSO_4$ electrophoresis the pellet was suspended in 400 μL of 30 mM Tris-Cl, pH 7.4, containing 5 mM EDTA, 0.6 M mercaptoethanol, 30 mM potassium chloride, and 100 mM sodium fluoride, $NaDodSO_4$ was added to a final concentration of 5%, and the solution was placed immediately in a water bath at 65 °C for 30 min. Between 50 and 150 μg of protein was applied per gel.

(2) Cardiac Sarcolemma. Cardiac sarcolemma was prepared by the procedure of St. Louis & Sulakhe (1976) from rat hearts immediately following dissection from the animal. Briefly, the hearts from three male Sprague-Dawley rats were removed following anesthesia induced by the injection of 30 mg/mL of Nembutal–1000 units/mL of heparin sulfate (4 mL/kg). All fractionation procedures were performed at 4 °C. The tissue was finely diced and homogenized in 10 mL of 10 mM Tris-Cl, pH 7.5, containing 2 mM dithiothreitol (TD buffer) for 2 s with a polytron tissue homogenizer (PT 10 ST, rheostat setting 9). To the homogenate was added 4 M potassium chloride (in TD buffer) to a final concentration of 1.25 M. The homogenate was stirred gently for 10 min at 0 °C and then centrifuged at 9000*g* for 10 min. The precipitate was suspended in 10 mL of 1.25 M KCl in TD buffer, stirred for 10 min, and collected by centrifugation at 4000*g*. This washing procedure was repeated twice with the precipitate suspended in TD buffer alone and collected by centrifugation at 3000*g*. The residue was suspended in 10% sucrose in TD buffer and 4 mL layered onto each of four Beckman SW 27 centrifuge tubes containing the following discontinuous sucrose gradients: 8 mL each of 45%, 50%, 55%, and 60% sucrose in TD buffer adjusted to pH 8.2. The tubes were centrifuged for 1 h at 40000*g*, the sample at the 50–55% sucrose interface was collected by aspiration and diluted threefold with TD buffer, and the membrane fraction was collected by centrifugation for 20 min at 11000*g*. The residue was suspended in 7 mL of 10% sucrose in TD buffer and layered on the top of two SW 27 centrifuge tubes containing the following discontinuous sucrose gradients: 8 mL each of 50%, 52.5%, 55%, and 60% sucrose in TD buffer adjusted to pH 8.2. The tubes were centrifuged for 1 h at 40000*g*, the fraction at the 52.5–55% interface was collected by aspiration and diluted threefold in TD buffer, and the enriched sarcolemma preparation was collected by centrifugation for 20 min at 11000*g*. The residue was suspended in 2 mL of 10% sucrose in TD buffer at a protein concentration between 1 and 2 mg/mL. Sarcolemma prepared by this procedure exhibits enzymological characteristics equivalent to those presented previously by St. Louis & Sulakhe (1976) and Hui et al. (1976). The ouabain-sensitive Na^+,K^+-ATPase and NaF-stimulated adenylylcyclase in the final fraction had specific activities of 10.1 and 0.213 μmol (mg of protein)$^{-1}$ h^{-1}, respectively; each represented a fourfold enrichment over the initial homogenate. The specific activities of total Na^+,K^+-ATPase and Mg^{2+}-dependent ATPase were 23.3 and 23.8 μmol (mg of protein)$^{-1}$ h^{-1}, respectively. Markers for other subcellular fractions of acid phosphatase (9.074 μmol (mg of protein)$^{-1}$ h^{-1}), NADPH-cytochrome *c* reductase (0.054 μmol (mg of protein)$^{-1}$ h^{-1}), and succinate dehydrogenase (2.36 μmol (mg of protein)$^{-1}$ min^{-1}) represented, respectively, a 5.4-, 6.3-, and 11.0-fold decrease in comparison to the homogenate; these

[1] The use of antiphosphorylase *b* kinase serum was to permit the isolation of ^{32}P-labeled phosphorylase kinase for an alternate study. This step is unnecessary for the isolation of sarcolemma proteins but was used in most of the experiments reported in this paper.

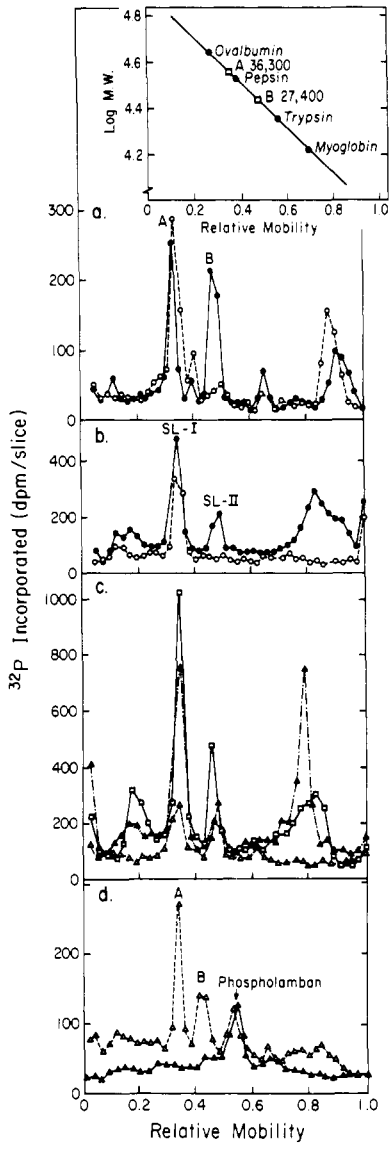

FIGURE 1: $NaDodSO_4$ gel electrophoretic profiles of cardiac membrane phosphoproteins. Gel electrophoresis was performed according to the method of Weber & Osborn (1969) with 10% acrylamide at pH 7.2 on 8-cm gels. Data are expressed in reference (R_m) to the migration of bromphenol blue. (a) Profile of the 50K fraction for either control (O---O) or hearts stimulated with 2 μM epinephrine for 60 s (●—●). Conditions as in Materials and Methods. (Insert) Molecular weight standardization curve. (b) Profile of phosphoproteins of the *enriched sarcolemma*. Sarcolemma at a protein concentration of 1.3 mg/mL was incubated at 30 °C for 2 min in the presence (●—●) or absence (O---O) of the catalytic subunit of cAMP-dependent protein kinase (20 μg/mL) in 45 mM Tris-Cl, pH 6.8, containing 120 mM potassium chloride, 5 mM magnesium sulfate, 20 mM sodium fluoride, 0.048 mM $CaCl_2$, and 0.084 mM EGTA. [γ-^{32}P]ATP (0.364 mCi/μmol) was added to a final concentration of 0.5 mM and the reaction mixture incubated for an additional 5 min. The reaction was terminated by the addition of $NaDodSO_4$ to a final concentration of 5%. The sample was immediately heated at 65 °C for 30 min prior to electrophoresis. (c) Comparison of the profiles of phosphoproteins obtained by the in vitro incubation of sarcolemma (▲·····▲), the 50K-fraction from perfused hearts (△---△), or the combination of both fractions (□—□). The 50K fraction was isolated as indicated in a from a heart stimulated for 20 s with 2 μM epinephrine. The phosphorylated enriched sarcolemma preparation was obtained as indicated in b except that the final ATP concentration was 2 mM obtained by equivalent additions at 0, 45, 90, and 135 s with a total incubation time of 3 min. (d) Comparison of the profiles of phosphoproteins obtained by the in vitro phosphorylation of sarcoplasmic reticulum (▲—▲) and of the combined fraction of sarcoplasmic reticulum and the 50K fraction from perfused hearts (△---△). The 50K fraction was obtained as indicated in c. The sarcoplasmic reticulum at a protein concentration of 2 mg/mL was incubated at 30 °C for 5 min in a 2.5 mM Tris-Cl, 2.5 mM oxalate, 40 mM histidine, 120 mM potassium chloride, 5 mM magnesium chloride, 20 mM sodium fluoride, 0.024 mM calcium chloride, and 0.084 mM EGTA, pH 6.8. Catalytic subunits of the cAMP-dependent protein kinase and [γ-^{32}P]ATP (0.26 mCi/μmol) were added to final concentrations of 10 μg/mL and 0.5 mM, respectively, and the reaction mixture was incubated for an additional 5 min. The reaction was terminated by the addition of $NaDodSO_4$ to a final concentration of 5%. The sample was heated at 65 °C for 30 min prior to electrophoresis.

values are equivalent to those obtained for other described preparations of cardiac sarcolemma (Hui et al., 1976; St. Louis & Sulakhe, 1976). The fraction prepared by this procedure with these characteristics has been designated "*enriched cardiac sarcolemma*" in this report.

(3) Cardiac Sarcoplasmic Reticulum. The enriched sarcoplasmic reticulum fraction was prepared from male Sprague-Dawley rats by the procedure of Harigaya & Schwartz (1969) with minor modifications as described by Kirchberger et al. (1974).

Other Assays. Marker enzymes for subcellular fractions were assayed by the following procedures: ATPase activities, Sulakhe et al. (1973); succinate dehydrogenase, King (1967); NADPH-cytochrome *c* reductase, Ragnotti et al. (1969); adenylate cyclase, St. Louis & Sulakhe (1976) with the exception that [^{32}P]cAMP was determined using the TLC system of Castagna et al. (1977); and acid phosphatase, Hubscher & West (1965) modified by the use of ascorbate for phosphate determination. Protein concentrations were determined by the method of Lowry et al. (1951). $NaDodSO_4$ gel electrophoresis was performed according to the procedure of Weber & Osborn (1969). At the termination of the electrophoresis, the gels were placed in 15% Cl_3CCOOH for 15 min at 0 °C, were stained with Coomassie Brilliant Blue, and were destained by washing in acetic acid–methanol–H_2O (7.5:50:42.5). The gels were permitted to swell in 7% acetic acid. Gels containing phosphorylated products were sliced transversely into 2-mm sections, and each section was placed on a piece (1 × 1.5 cm) of Whatman No. 31 ET chromatography paper, dried for 1 h at 115 °C, and counted in 5 mL of toluene-based scintillation fluor containing 4 g/L of Omnifluor (New England Nuclear).

Materials. α-Amylase (type II-A) (870 units/mg) was purchased from Sigma Chemical Co. and pretreated by incubation at 30 °C for 30 min with 1 mM phenylmethanesulfonyl fluoride. Anti-phosphorylase kinase serum was prepared against rabbit skeletal muscle phosphorylase kinase isolated by the procedure of Hayakawa et al. (1973). Homogeneous bovine skeletal muscle cAMP-dependent protein kinase catalytic subunit was prepared by the procedure of Beavo et al. (1974). The heat-stable inhibitor protein of the cAMP-dependent protein kinase and [γ-^{32}P]ATP were prepared as described previously (Walsh et al., 1971a,b). Chymotrypsin, pronase, and subtilisin were purchased from Worthington, Calbiochem. Corp., and Nutritional Biochemical Co., respectively.

Results

Characterization of Cardiac Membrane Phosphoproteins. The phosphorylation of proteins of the 50K fraction of cardiac membranes was determined by $NaDodSO_4$ gel electrophoresis for both control and epinephrine-stimulated tissue. For tissue isolated following a 30-min equilibration period with inorganic [^{32}P]phosphate the 50K fraction contains one major (designated A) and several minor phosphoproteins (Figure 1a). Stimulation of the heart with 2 μM epinephrine promoted the phosphorylation of a second major protein (designated B). From a sequence of experiments (not shown) it was established

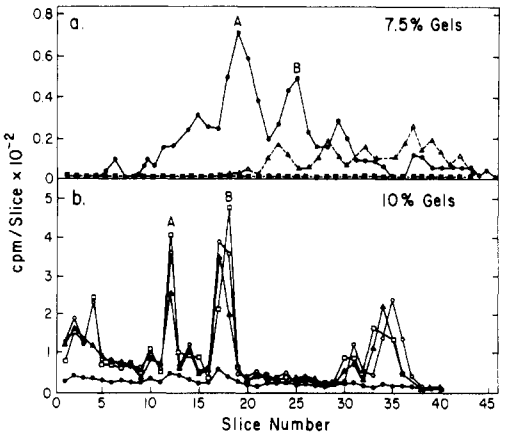

FIGURE 2: Characterization of the phosphorylated products of the 50K fraction obtained from perfused rat hearts. (a) Effect of proteolytic enzymes. The 50K fraction from hearts stimulated with 1 μM epinephrine for 90 s was prepared as indicated for Figure 1a. The sample, as extracted in $NaDodSO_4$ sample buffer (Weber & Osborn, 1969) containing 0.180 mg of protein, was incubated with 10 μg of either subtilisin (□—□), Pronase (▲—▲), chymotrypsin (△---△), or no additions (●—●) for 20 min at 30 °C. The subtilisin incubation also contained 1.3 mM calcium chloride. Conditions of electrophoresis are identical with those of Figure 1 with the exception that the gels were 7.5% acrylamide. (b) Chemical stability of the phosphoproteins. The 50K fraction obtained from hearts stimulated with 2 μM epinephrine for 60 s was incubated with (●—●) 0.5 N NaOH at 65 °C for 20 min, (▲—▲) 10% Cl_3CCOOH at 65 °C for 20 min, or (□—□) 0.8 M hydroxylamine plus 0.05 M sodium acetate, pH 5.4, at 30 °C for 20 min. ○—○ indicates the untreated control. At the termination of incubations 2 volumes of ice-cold 20% Cl_3CCOOH was added to each incubation, the mixtures were centrifuged at 1500g for 5 min, the pellets were washed twice with cold distilled water and the final pellet was solubilized in $NaDodSO_4$ as described in Materials and Methods. Conditions of electrophoresis are identical with Figure 1.

that no significant change in the amount of phosphate in the A protein occurred in response to epinephrine. This report is concerned with a study of the A and B proteins; the amount of ^{32}P in other fractions has been less consistent between experiments and has not been further investigated. The [^{32}P]phosphate of the A and B bands has been designated as protein bound on the basis of sensitivity to the proteolytic enzymes chymotrypsin, Pronase, and subtilisin (Figure 2a). The ^{32}P-phosphate bond of both the A and B proteins was labile in 0.5 N sodium hydroxide but stable to treatment with either 0.8 M hydroxylamine or 10% Cl_3CCOOH (Figure 2b). The latter are characteristics of a phosphoester bond (phosphoserine or phosphothreonine). The molecular weights of the A and B proteins, determined by comparison with standards (inset, Figure 1a), are 36 300 and 27 400, respectively.

In consideration of the reports of Krause et al. (1975) and Kirchberger et al. (Kirchberger et al., 1974; Kirchberger & Chu, 1976) of proteins of molecular weight 24 000 and 22 000 from cardiac sarcolemma and sarcoplasmic reticulum, respectively, each of which has been shown to be a substrate in vitro of the cAMP-dependent protein kinase, we have evaluated the potential identity of each of these latter proteins with the B protein, of the 50K fraction isolated from perfused heart. In Figure 1b is presented the $NaDodSO_4$ gel electrophoresis profile of phosphoproteins of *enriched cardiac sarcolemma*; the characteristics of this latter preparation have been described in detail by Sulakhe et al. (1973, 1976). Following incubation of the rat heart sarcolemma preparation with [γ-^{32}P]ATP, a single major protein (designated SL-I) of mol wt 36 000–37 000 is identified; the phosphorylation of this protein is presumably catalyzed by a protein kinase endogenous

Table I: Effect of the Heat-Stable Inhibitor Protein of the cAMP-Dependent Protein Kinase on Protein Phosphorylation of Enriched Sarcolemma[a]

additions		phosphate incorp (pmol of ^{32}P incorp/mg of protein)	
catalytic subunit	inhibitor protein	SL-I	SL-II
–	–	9.1	<0.7
–	+	9.5	<0.7
+	–	23.7	6.6
+	+	8.7	<0.7

[a] The phosphorylation of proteins of enriched sarcolemma was determined as described in the legend of Figure 1b in the presence or absence of 9280 units/mL of the heat-stable inhibitor protein and/or 33 μg/mL of catalytic subunit of the cAMP-dependent protein kinase as indicated. At the termination of the reaction, the extent of ^{32}P incorporated into SL-I and SL-II was calculated from the summation of each peak of the $NaDodSO_4$ gel electrophoresis profile.

Table II: Comparison of the Mobilities of Phosphoproteins of Enriched Sarcolemma Phosphorylated in Vitro and the 50K Fraction Phosphorylated in the Perfused Heart[a]

	R_m values	
	peak A (SL-I)	peak B (SL-II)
perfused heart 50K fraction	0.34 ± 0.03 (73)	0.48 ± 0.03 (70)
in vitro phosphorylated sarcolemma	0.35 ± 0.02 (27)	0.47 ± 0.04 (15)

[a] Conditions of the experiment are identical with those presented for Figures 1a and 1b. Values are means ± SD for the number of determinations in parentheses.

to the preparation. This latter kinase is not inhibited by the heat-stable inhibitor protein of the cAMP-dependent protein kinase (Table I) and thus is neither the cAMP-dependent protein kinase nor the catalytic subunit derived therefrom. Addition of catalytic subunit of the cAMP-dependent protein kinase to the sarcolemma preparation increased the phosphate incorporation into SL-I approximately twofold, but in addition catalyzed the phosphorylation of a second protein (designated SL-II) of molecular weight 27 000 (Figure 1b). The addition of heat-stable inhibitor protein blocked the catalytic subunit catalyzed phosphorylation of SL-II and reduced the phosphorylation of SL-I to that level catalyzed by the endogenous kinase (Table I). SL-II is presumably identical with the sarcolemma protein characterized by Krause et al. (1975) of molecular weight quoted as 24 000, the phosphorylation of which is presumably associated with the enhanced calcium transport described by Will et al. (1973), Sulakhe et al. (1976), and Hui et al. (1976). In Figure 1c and Table II is presented a comparison of SL-I and SL-II phosphoproteins with the A and B proteins of the 50K fraction obtained from perfused heart. As indicated the A protein comigrates with SL-I and the B protein comigrates with SL-II.

By a similar experimental approach a comparison has been made between the phosphoproteins of the 50K fraction from perfused heart and phospholamban, the cAMP-dependent protein kinase stimulated phosphoprotein of cardiac sarcoplasmic reticulum (Kirchberger et al., 1974, 1976; Tada et al., 1974, 1975). As indicated (Figure 1d), phospholamban exhibited a molecular weight between 20 700 and 22 000 consistent with the value of 22 000 repeatedly documented by Kirchberger et al. (1974, 1976). Phospholamban was readily separated from the B protein of the 50K fraction.

In Figure 3 is presented the profile of proteins of the 50K fraction isolated from hearts perfused with nonradioactive

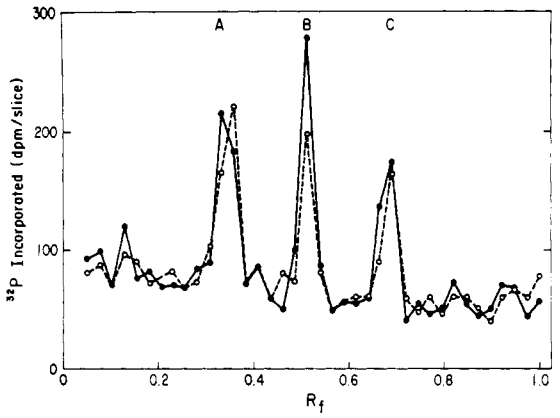

FIGURE 3: NaDodSO$_4$ gel electrophoresis profile of proteins of the 50K fraction from perfused hearts phosphorylated in vitro. Hearts were perfused as indicated in Materials and Methods with the exception that no inorganic [^{32}P]phosphate was added to the recirculating perfusion media. The 50K fraction was isolated by the standard procedure and the final pellet was suspended in 50 mM potassium chloride, 20 mM Tris-Cl, 20 mM maleate, pH 6.8. The membrane fraction at a protein concentration of 3.2 mg/mL was incubated at 30 °C for 5 min in 45 mM Tris-Cl, 120 mM potassium chloride, 5 mM magnesium sulfate, 20 mM sodium fluoride, 0.048 mM calcium chloride, 0.084 mM EGTA, pH 6.8. Either [γ-^{32}P]ATP (0.093 mCi/μmol) (○—○) or [γ-^{32}P]ATP plus the catalytic subunit of the cAMP-dependent protein kinase (●—●) was added to final concentrations of 0.5 mM and 80 μg/mL, respectively, and the reaction mixture incubated at 30 °C for an additional 5 min. The reaction was terminated by the addition of NaDodSO$_4$ to a final concentration of 5% and the sample immediately heated at 65 °C for 30 min. Conditions of electrophoresis are identical with those of Figure 1.

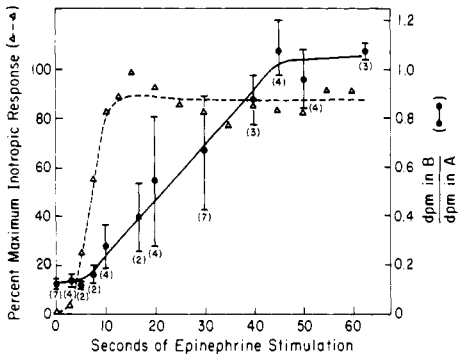

FIGURE 4: Time course of the phosphorylation of the B protein of the 50K fraction of perfused heart in response to stimulation with 2 μM epinephrine. Conditions of perfusion are given in Materials and Methods. The extent of ^{32}P incorporation was determined from NaDodSO$_4$ gel electrophoresis profiles performed according to the conditions of Figure 1a. The bars represent the mean ± SD calculated from the number of hearts indicated in brackets. For values less than 3, the bars indicate the average and the range of values.

phosphate and phosphorylated in vitro by incubation with [γ-^{32}P]ATP. Three phosphoproteins are evident, two of which correspond to the A and B bands phosphorylated in the intact heart. The third band (designated C) comigrated with a minor band often detected in the perfused heart preparation (cf. Figure 1a). The addition of catalytic subunit of the cAMP-dependent protein kinase enhanced the phosphorylation of the B band but was without effect on that of A or C. A sample of the 50K fraction, phosphorylated in vitro, was added back to an extract of heart perfused with nonradioactive media and the 50K fraction reisolated. A quantitative recovery of the phosphoproteins indicated that no significant phosphoprotein phosphatase activity was occurring during the isolation procedure used to determine the in vivo phosphorylated membrane proteins (data not presented).

Regulation of Sarcolemma Protein Phosphorylation in the

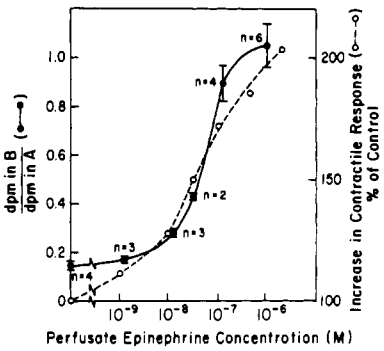

FIGURE 5: Dose–response curve for epinephrine stimulation of the phosphorylation of the B protein of the 50K fraction. Hearts were perfused with epinephrine at the concentrations indicated with nonrecirculating medium for 60 s. The bars represent the mean ± SD calculated from the number of hearts indicated in brackets. For values less than 3, the bars indicate the average and the range of values.

Table III: Characteristics of Hormonal Response for the Phosphorylation of Proteins of the 50K Fraction in Perfused Rat Heart[a]

	protein phosphorylation dpm B/dpm A	
	treatment	− Epi
expt 1	none	0.15 ± 0.01
	propranolol, 10 μM	0.18 ± 0.01
	phenoxybenzamine, 10 μM	0.19 ± 0.14
	epinephrine, 1 μM	1.05 ± 0.23
	plus propranol	0.19 ± 0.03
	plus phenoxybenzamine	0.94 ± 0.05
expt 2	none	0.15 ± 0.01
	DL isoproterenol, 1 μM	0.94 ± 0.03
	phenylephrine, 1 μM	0.15 ± 0.01
expt 3	none	0.15 ± 0.01
	glucagon (1×10^{-7} M, 60 min)	0.70 ± 0.04
	glucagon, 0.1 μM (1×10^{-7} M, 90 min)	1.23 ± 0.20

[a] The 50K fraction was isolated by the conditions indicated in Materials and Methods from hearts perfused under the indicated conditions of hormonal stimulation. The extent of phosphorylation of the B protein (vide Figure 1) is presented as the ratio of dpm in B:dpm in A as indicated in Figure 4. For experiment 1 the hearts were perfused with the catecholamine antagonists for 60 s prior to epinephrine addition. Stimulation with epinephrine, α or β agonists, was for 60 s. The values are for a minimum of three determinations for each of 2–3 animals per group; n = total number of determinations.

Perfused Rat Heart. Evidence presented in Figure 1a showed that the phosphorylation of the B protein of the 50K fraction was increased in the perfused heart in response to epinephrine. In Figure 4 is presented a time course of this event. In repeated experiments no change has been detected in the incorporation of ^{32}P into the A protein per mg of protein in response to epinephrine and, in consequence, quotation of the data on the basis of the ratio dpm in B/dpm in A is a measure of phosphorylation of B and provides an internal control that compensates both for differences within individual experiments in tissue specific activities of [γ-^{32}P]ATP and of recoveries of membrane fraction during isolation. The data presented in Figure 4 illustrate the time course of phosphorylation of the B protein in response to epinephrine. It is apparent that the phosphorylation of the B protein is not the mediator of the onset of the inotropic effect. It is of interest that at maximum incorporation there was a 1:1 stoichiometry in the A and B proteins possibly suggesting a specific molar ratio of these two proteins. The response to epinephrine for the phosphorylation of the B protein of the 50K fraction occurs within the

Table IV: Characteristics of the Phosphoproteins of the 50K Fraction of Perfused Rat Heart Following Removal of Catecholamine Stimulation[a]

period of wash following epinephrine stimulation (s)	dpm B/dpm A	^{32}P incorp (dpm/mg of 50K protein × 10^{-3})	
		A protein	B protein
0	0.49 ± 0.04	25.6	12.4
40	0.48 ± 0.03	27.6	13.2
80	0.43 ± 0.03	30.8	13.9
120	0.64 ± 0.03	20.3	13.3
160	0.65 ± 0.03		

[a] Hearts were perfused with ^{32}P as described in Materials and Methods and perfused with 0.1 μM epinephrine for 40 s. Perfusion was continued by drip-through for the times indicated with control Krebs-Henseleit media. The values are for a minimum of three determinations for each of 2-3 animals per group; n = total number of determinations. Catecholamine stimulation increased contractile response to 73 ± 15% above control. Washout for 40 s or greater decreased the contractile response to basal ± 10%.

physiological concentration range for the catecholamine (Figure 5), half-maximal stimulation occurring at 0.2 μM, a value comparable to that of epinephrine-induced inotropic effect. The epinephrine response is characteristic for interaction with the β receptor; epinephrine-stimulated phosphorylation of the B protein is blocked by propranolol but not by phenoxybenzamine and can be mimicked by isoproterenol but not by phenylephrine (Table III). The phosphorylation of the B protein is also stimulated by glucagon (Table III); the effect required a longer time than that for catecholamines to reach maximum but glucagon-stimulated inotropic response also requires a longer time to reach maximum in comparison with epinephrine. We have examined the dephosphorylation of the B protein of the 50K fraction following the removal of the epinephrine stimulus. The experiment presented in Table IV was performed under conditions in which catecholamine-stimulated phosphorylation of the B protein was submaximal (epinephrine, 0.2 μM; 40-s stimulation; B/A = 0.49; cf. Figures 4 and 5). As indicated (Table IV) the amount of ^{32}P in protein B remained constant (at the submaximal level) for 160 s following the removal of epinephrine; a slight elevation in the ratio of ^{32}P in the B:A proteins could be attributed to a small loss of ^{32}P phosphate from the A protein. (The latter is an incongruous observation since the phosphorylation of the A protein is not apparently enhanced by epinephrine.) The conditions of the experiment of Table IV were more than adequate to remove epinephrine from the system; both the inotropic response and phosphorylase kinase activity decrease to basal level within 60 s (McCullough & Walsh, unpublished observation).

Discussion

Recent studies of the subcellular ultrastructure of cardiac muscle and of the effects of the rare earth cation lanthanum on the contractile response, and evidence from electrophysiological techniques, have provided important documentation of the role of sarcolemma in the regulation of cardiac contraction. These approaches have emphasized that, especially in comparison with skeletal muscle, the major source of Ca^{2+} to stimulate contraction of cardiac muscle is derived from the extracellular space (for a review, see Langer, 1973). In consequence, the regulation of Ca^{2+} transport across cardiac sarcolemma is potentially a prime site of control of contractile events. Studies of Will et al. (1973), Sulakhe et al. (1976), St. Louis & Sulakhe (1976), Krause et al. (1975), and Hui et al. (1976) have provided in vitro evidence that cAMP, as mediated by the protein kinase, may regulate the transport of Ca^{2+} across the sarcolemma. Thus it is a viable hypothesis that one site of action whereby catecholamines modulate cardiac contraction is via the stimulation of the phosphorylation of cardiac sarcolemma protein(s).

The standard preparations of sarcolemma so far devised (Sulakhe et al., 1976; Hui et al., 1976; Krause et al., 1975) require as a starting material tissue that has not been frozen. Thus, with these preparations, it is not possible to examine rapid phosphorylation–dephosphorylation events as they may occur in the intact cell in response to epinephrine. This current study reports the isolation from freeze-clamped hearts of a membrane fragment apparently derived from the sarcolemma. This fragment contains two phosphoproteins that are similar, or identical, to two proteins present in the enriched sarcolemma preparation. This comparison and identification are based not only on the molecular size of the proteins but also on the characteristics of phosphorylation. Thus the A protein (i.e., SL-I) is phosphorylated in vivo (Figure 1a) in the absence of a cAMP signal (presumably by a cAMP-independent kinase) and in vitro (Figure 1b and Table I) by an endogenous cAMP-independent kinase present in the membrane. Similarly the B protein (i.e., SL-II) is phosphorylated in response to a cAMP signal both in vivo (Figure 1a and Table IV) and in vitro (Figure 1b and Table II). Also of importance, the maximum amount of phosphorylation of SL-II from long-term stimulation (56 ± 11 μmol/mg of protein, n = 6) is equivalent to that incorporated in vitro (42 μmol/mg of protein, conditions of Figure 1b). One caution that must be exercised in interpreting both this and other studies is that the preparation used for the in vitro studies can at best be described as "enriched sarcolemma", thus leaving open the possibility that these phosphoproteins are associated with a contaminant. Nevertheless, the sarcolemma preparation does exhibit modified Ca^{2+} transport in response to cAMP-stimulated protein kinase (Sulakhe et al., 1976; Hui et al., 1976; Krause et al., 1975) and the data presented in Figure 1 (b and d) show that the sarcolemma preparation is not significantly contaminated with sarcoplasmic reticulum membrane containing phospholamban. A stimulation of A protein phosphorylation by cAMP is also observed in vitro (Figure 1b) but not in vivo, possibly representing a multisite phosphorylation analogous to the type of regulation that is becoming apparent with other systems (Cohen & Antoniw, 1973; Soderling et al., 1977).

England (1975, 1976) has demonstrated not only that the phosphorylation of cardiac troponon I occurs in response to catecholamines but also that there is a substantial correlation between this phosphorylation and the onset of the inotropic effect. Clearly the phosphorylation of troponin is a primary candidate as the mediator of epinephrine-enhanced contraction whereas, in contrast, the phosphorylation of the B protein occurs after the maximum stimulation of contraction has been reached (Figure 4) and is not diminished upon removal of the epinephrine stimulus under conditions in which the contractile response has returned to basal (Table IV). Thus if the phosphorylation of the B protein plays a role in the regulation of contraction, its involvement is subtle at best. Interestingly, England's data (1976) showed that there was not a correlation between dephosphorylation of troponin and the decrease of the inotropic effect following the removal of isoproterenol. It is becoming increasingly apparent that multisite phosphorylation of a single protein occurs (Cohen & Antoniw, 1973; Nimmo et al., 1976; Schlender & Reimann, 1975; Soderling et al., 1977) and that the phosphorylation at different sites modulates different parameters; in the case of phosphorylase kinase, phosphorylation by the cAMP-dependent protein kinase

not only initiates the activation of the enzyme but also apparently regulates its inactivation (Cohen & Antoniw, 1973). With this as a model, it would not be surprising that the regulation of an event as complex as contraction occurs by the phosphorylation of more than one protein and that separate phosphorylation events may modulate the initiation and the duration of the event. Thus the phosphorylation of troponin, sarcolemma proteins, and phospholamban could all be components of the mechanism by which cAMP regulates contraction, but any direct correlation between the phosphorylation of sarcolemma proteins, an associated change in Ca^{2+} uptake, and the regulation of contraction is not readily apparent.

Independent of any potential role of the B protein of the 50K fraction in the regulation of contraction, the data presented in this paper emphasize a fundamental concept of cAMP function that remains to be elucidated. The cAMP-dependent protein kinase is a unique intracellular enzyme in that it is a single species of catalytic function but with multiple substrates. Essentially no information is available to permit evaluation of the preferential order of protein substrate phosphorylation that occurs in response to a cAMP signal within the cell. In the perfused heart, under conditions as described herein, an epinephrine-induced intracellular increase in cAMP results initially in the phosphorylation of phosphorylase kinase ($t_{1/2}$ = 5 s; McCullough & Walsh, unpublished observation). Troponin, following an initial 5-s lag, is maximally phosphorylated by 25 s ($t_{1/2}$ = 15 s), whereas the B sarcolemma protein is not phosphorylated maximally until 45 s. The phosphorylation of each of these proteins is catalyzed by the cAMP-dependent protein kinase; yet obviously the presence or absence of cAMP is not the sole determining factor that regulates these processes. What these additional regulatory phenomena are is of obvious importance to the full understanding of the mechanisms by which hormones control cellular processes.

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