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CYCLIC AMP STIMULATION OF MEMBRANE PHOSPHORYLATION AND Ca^{2+}-ACTIVATED, Mg^{2+}-DEPENDENT ATPase IN CARDIAC SARCOPLASMIC RETICULUM*

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SUMMARY

Ca^{2+}-activated ATPase (EC 3.6.1.15) in canine cardiac sarcoplasmic reticulum was stimulated 50–80 % by cyclic adenosine 3′ : 5′-monophosphate. The relationship of this stimulation to cyclic AMP-dependent membrane phosphorylation with phosphoester bands was studied. Cyclic AMP stimulation of ATPase activity was specific for Ca^{2+}-activated ATPase and was half-maximal at about 0.1 μM which is similar to the concentration required for half-maximal stimulation of membrane phosphorylation by endogenous cyclic AMP-stimulated protein kinase (EC 2.7.1.37). Cyclic AMP stimulation of Ca^{2+}-activated ATPase was calcium dependent and maximal at calculated Ca^{2+} concentrations of 2.0 μM. Cyclic AMP-dependent Ca^{2+}-activated ATPase correlated well with the cyclic AMP-dependent membrane phosphorylation of which 80 % was 20 000 molecular weight protein identified by sodium dodecyl sulfate discontinuous polyacrylamide gel electrophoresis. In trypsin-treated microsomes, cyclic AMP did not stimulate Ca^{2+}-activated ATPase or phosphorylation of the 20 000 molecular weight membrane protein. An endogenous calcium-stimulated protein kinase (probably phosphorylase *b* kinase) with an apparent K_m for ATP of 0.21–0.32 mM was present and appeared to be involved in the cyclic AMP-dependent phosphorylation of the 20 000 molecular weight protein which was calcium dependent. Cyclic guanosine 3′ : 5′-monophosphate did not inhibit any of the stimulatory effects of cyclic AMP. These data suggest that the cyclic AMP stimulation of Ca^{2+}-activated ATPase in cardiac sarcoplasmic reticulum is mediated by the 20 000 molecular weight phosphoprotein product of a series of kinase reactions similar to those activating phosphorylase *b*.

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid; SDS, sodium docedyl sulfate.

* In conducting the research described in this report, the investigators adhered to the 'Guide for the Care and Use of Laboratory Animals', DHEW publication No. (N.I.H.) 73-23, as prepared by the Institute of Laboratory Animal Resources, National Research Council.

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INTRODUCTION

Cyclic AMP as well as activators of endogenous adenylate cyclase increases oxalate-dependent calcium uptake in some cardiac sarcoplasmic reticulum [1, 2]. Cyclic AMP also stimulates membrane phosphorylation in cardiac sarcoplasmic reticulum [2, 3]. As we previously demonstrated, an endogenous cyclic AMP-stimulated protein kinase (EC 2.7.1.37) and a substrate are associated with cardiac sarcoplasmic reticulum, suggesting that the phosphoprotein product mediates the cyclic AMP effect on calcium uptake by regulating Ca^{2+}-activated ATPase (EC 3.6.1.15) [4]. In another sarcoplasmic reticulum preparation exogenous protein kinase is required to achieve cyclic AMP stimulation of oxalate-dependent calcium uptake [5]. In that system exogenous protein kinase in the presence of cyclic AMP also increases Ca^{2+}-activated ATPase [6].

To further understand the regulatory role of cyclic AMP in calcium transport by sarcoplasmic reticulum, we investigated the effect of cyclic AMP on Ca^{2+}-activated ATPase in a canine cardiac sarcoplasmic reticulum preparation previously demonstrated to contain cyclic AMP-stimulated protein kinase and a substrate. This paper reports the cyclic AMP stimulation of Ca^{2+}-activated ATPase in these microsomes and describes our investigation of the relationship between cyclic AMP-dependent membrane phosphorylation and cyclic AMP stimulation of Ca^{2+}-activated ATPase. Portions of this work have been published in abstract form [7–9].

METHODS

Materials. Aquasol, Triton X-100, $K_2H^{32}PO_4$ with 1 Ci/mmol and [γ-^{32}P]-ATP, tetra(triethylammonium) salt with 10–40 Ci/mmol, were obtained from New England Nuclear, Boston, Mass. Papaverine · HCl was purchased from S. B. Penick and Company, New York, N.Y. All non-radioactive nucleotides, ouabain and hog stomach mucosa pepsin were bought from Sigma Chemical Company, St. Louis, Mo. Sperm whale myoglobin, trypsin, ovalbumin, bovine serum albumin, human γ-globulin and chymotrypsinogen were obtained from Schwarz/Mann, Orangeburg, N. J. Phosphorylase *a* and β-lactoglobulin was bought from Calbiochem, LaJolla, Calif. All other chemicals were reagent grade and obtained from local suppliers.

Preparation of cardiac microsomes. Canine cardiac sarcoplasmic reticulum was prepared by a modification of the method of Katz and Repke [10]. Ice-chilled ventricular muscle removed from pentobarbital-anesthetized adult dogs was immediately diced and homogenized in a Waring blender on high speed for 20 s twice at 5–10 °C in 2.5 volumes of 0.3 M sucrose, 5 mM Tris/oxalate, 0.2 mM α-tocopherol and 5 mM histidine, pH 7.4. After centrifugation for 30 min at a maximal force of 13 500 $\times g$ the supernatant was filtered through four layers of cheese cloth and centrifuged for 90 min at a maximal force of 180 000 $\times g$. After decanting the supernatant, the pellet was taken up in 6–9 ml of the original homogenizing solution with 1 mM Tris/oxalate and gently homogenized by hand. The suspension was sedimented on a discontinuous gradient of 20–35 % sucrose at a maximal force of 78 000 $\times g$ for 90 min. The membranes at the interface were collected. This procedure yielded between 0.09 and 0.13 mg protein/g wet weight of cardiac muscle. Aliquots of this preparation with 0.4–1.0 mg protein/ml were stored for up to 4 days at −10 °C in 0.8 M sucrose, 0.2 mM α-

tocopherol and 2 mM dithiothreitol without loss of the various ATPase or cyclic AMP-stimulated protein kinase activities. Membrane proteins were determined by the method of Lowry et al. [11] with bovine serum albumin as the standard. All enzymatic activities were expressed in terms of mg of membrane protein.

ATPase assay. ATPase activities were routinely determined by incubating membranes at 37 °C for 30 min in a final volume of 0.5 ml with 0.06–0.12 mg of protein/ml, 10 mM histidine, pH 7.0, 120 mM KCl, 0.2 M sucrose, 0.04 mM α-tocopherol, 0.4 mM dithiothreitol and 5 mM Mg_2ATP with various concentrations of $CaCl_2$. The reaction was started by addition of the membranes and terminated by addition of 0.1 ml of cold 50 %(w/v) trichloroacetic acid. The P_i in the $3000 \times g$ supernatant was measured by the method of Bernhart and Wreath [12]. Blanks of 20–40 nmol of P_i were obtained by adding ATP or membranes after the acid denaturation. Each reaction was incubated in triplicate. Ca^{2+}-activated ATPase was calculated by subtracting basal ATPase assayed in the presence of 0.5 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA) from the ATPase in the presence of $CaCl_2$. The data from single experiments shown in Figs. 1–3 were confirmed in at least two other experiments.

Determination of membrane phosphorylation. Membrane phosphorylation was determined under the condition of the ATPase assay using a slight modification of methods previously described [4]. The final volume was 0.2 ml with [γ-^{32}P]ATP added to obtain 5–10 cpm/pmol of total ATP. The reaction was terminated by adding 4 ml of cold 10 % (w/v) trichloroacetic acid containing 0.5 mM Na_2ATP and 0.5 mM KH_2PO_4 immediately followed by 0.2 ml of 0.63 % (w/v) bovine serum albumin. The precipitate was dissolved in 1 M NaOH at room temperature and immediately reprecipitated with the above trichloroacetic acid solution a total of three times.

When the initial trichloroacetic acid precipitate was washed three times with only the trichloroacetic acid solution, the bound ^{32}P in the precipitate was increased by as much as 0.6 nmol/mg protein of which the majority was not bound to membrane components of $M_r > 15\,000$ as determined by gel electrophoresis described below. A smaller part of the ^{32}P was bound in the 90 000–100 000 molecular weight region of the gels. The three membrane components discussed in this paper were present in slightly greater amounts (15–20 % increase) than when NaOH was used to dissolve the precipitates.

The endogenous protein kinase activity was determined in a reaction mixture of 0.25 ml of 50 mM sodium β-glycerophosphate, pH 7.0, 10 mM NaF, 2 mM theophylline, 0.3 M sucrose, 0.08 mM α-tocopherol, 0.8 mM dithiothreitol and 15 mM $MgCl_2$ with varying concentrations of [γ-^{32}P]ATP and $CaCl_2$. The [γ-^{32}P]ATP was added to obtain 10–70 cpm/pmol of total ATP. This reaction was performed at 30 °C for 1 min and terminated as described above. Endogenous protein kinase activity was linear from 30 to 60 s with 0.025–0.5 mg of protein/ml, 20 or 400 μM [γ-^{32}P]ATP and 0.5 mM EGTA or 10 μM $CaCl_2$ with and without 10 μM cyclic AMP. All phosphorylations were performed in duplicate.

Characterization of phosphorylated membranes. Phosphorylation of membranes was performed in duplicate under the conditions of the ATPase assay with 100 μM $CaCl_2$ or with 10 μM $CaCl_2$ and 25 μM cyclic AMP as well as under the conditions of the endogenous protein kinase assay with 25 μM cyclic AMP. Phosphorylated membranes were initially collected either by four trichloroacetic acid

precipitations described above or by centrifugation at a maximal force of 180 000 $\times g$ for 30 min. The trichloroacetic acid precipitates were resuspended and incubated for 15 min in 2 ml of various solutions before being reprecipitated in cold 10 % (w/v) trichloroacetic acid and counted for ^{32}P as described above. The following solutions were used: (1) 10 % (w/v) trichloroacetic acid at 0 °C, (2) 10 % (w/v) trichloroacetic acid at 90 °C, (3) ethanol/ethyl ether/chloroform (2 : 2 : 1, v/v) at 20 °C, (4) 150 mM sodium β-glycerophosphate, pH 7.0 at 90 °C, (5) 1 M NaOH at 90 °C, (6) 0.8 M NaCl, 0.1 M sodium acetate, pH 5.5 at 30 °C, (7) 0.8 M hydroxylamine, 0.1 M sodium acetate, pH 5.5, at 30 °C. The membrane pellets were resuspended and incubated at 0 °C for 30 min in 2 ml of 50 mM sodium β-glycerophosphate, pH 7.0, or 1 mg/ml Triton X-100. Membrane pellets were also washed twice at 25 °C in 1.5 ml of 50 mM sodium β-glycerophosphate, pH 7.0, or in the same buffer plus 0.6 M KCl. After recentrifugation the membrane pellets were acid precipitated four times and counted as described above.

Gel electrophoresis of phosphorylated membranes. Membranes were incubated for 30 min under the conditions of the ATPase assay in a total volume of 3.0 ml with [γ-^{32}P]ATP added to obtain 60–600 cpm/pmol of total ATP. 30 s prior to termination of the reaction 15-μl aliquots of the mixture were taken for determination of membrane phosphorylation as described above. The reactions were terminated by the addition of 3 ml of cold 20 % (w/v) trichloroacetic acid containing 1 mM Na_2ATP and 1 mM KH_2PO_4. After three subsequent precipitations identical to those performed on the 15-μl aliquots, the precipitates were washed with 2 ml of acetone and dissolved in 0.3–0.5 ml of 2 % (w/v) sodium dodecyl sulfate (SDS), 1 % (w/v) mercaptoethanol, 10 % (w/v) glycerol and 0.002 % (w/v) bromphenol blue in 0.06 M Tris · HCl, pH 8.0. Solutions were then adjusted to pH 7.5. After standing overnight at room temperature, the mixtures were heated at 100 °C for 1 min. An aliquot was taken for determination of total ^{32}P and 0.2–0.3 ml of the 2 % (w/v) SDS mixtures containing an estimated 0.09–0.20 mg of protein was applied to a 0.1 % (w/v) SDS discontinuous polyacrylamide electrophoresis system for acidic substances using a 10 cm 13 % (w/v) acrylamide resolving gel [13]. After electrophoresis the gels were sectioned at the dye front. The portions from the origin to the dye front were stained and destained according to the methods of Weber and Osborn [14] except that staining was done for 16 h using 0.025 % (w/v) Coomassie Brilliant Blue. Gel scannings to determine the relative absorbance at 550 nm were performed using whole gels or longitudinally sectioned half gels. After freezing, the gels were sliced in 2-mm sections. Each section was placed in a counting vial with 1 ml of 30 % (w/v) H_2O_2 and heated at 70 °C for 3 h to dissolve the gel. After cooling the vial and adding 10 ml of Aquasol, the ^{32}P was determined by liquid scintillation counting with correction for radioactive decay. Preliminary experiments under conditions of the ATPase assay using 5 mM Mg_2ATP or 1 mM Mg_2ATP and 8 mM $MgCl_2$ revealed no differences in membrane phosphorylation or in ^{32}P patterns in the SDS gels. All gel electrophoretic experiments reported here were performed with 1 mM Mg_2ATP.

Apparent molecular weights of the membrane proteins were estimated by comparison of their mobilities to the mobilities of protein standards in the 0.1 % (w/v) SDS discontinuous polyacrylamide electrophoresis system. The molecular weights of protein standards were those used by Weber and Osborn [14] except for those of the heavy and light chains of human γ-globulin [15]. Preliminary experiments demon-

strated that protein standards which were precipitated with trichloroacetic acid and washed with acetone had mobilities identical to the mobilities of standards which were only dissolved in the 2 % (w/v) SDS solution described above; therefore, protein standards were routinely run without prior precipitation.

Trypsin treatment of microsomes. Microsomes (4 mg/ml) were treated for 20 min at 25 °C with trypsin in the presence of 1 M sucrose as described by Tada et al. [16].

RESULTS

Characterization of cardiac microsomes

The sarcoplasmic reticulum preparation was characterized for contamination by enzymatic markers of other membrane structures as well as for the enzymatic activities related to the metabolism and possible action of cyclic AMP. We have previously shown that this sarcoplasmic reticulum preparation lacks significant activities of some marker enzymes for mitochondria and sarcolemma [4]. Ouabain-sensitive $(Na^+ + K^+)$-ATPase, another enzymatic marker of sarcolemma [17], was low (0.58 ± 0.15, μmol/mg protein per 30 min, mean $\pm$S.E., $n = 4$) representing less than 9 % of the total $(Na^+ + K^+)$-ATPase of these membranes which was only slightly higher than the basal ATPase in the absence of Na^+ (Table I). Azide-sensitive basal ATPase

TABLE I

ATPase ACTIVITIES ASSOCIATED WITH SARCOPLASMIC RETICULUM

ATPase activities in four sarcoplasmic reticulum preparations were assayed as described in Methods and expressed as means $\pm$S.E. $(Na^+ + K^+)$-ATPase was determined with optimal Na^+ and K^+ concentrations [17]. Basal ATPase was assayed in the presence of 0.5 mM EGTA. Ca^{2+}-activated ATPase was calculated by subtracting basal ATPase from the ATPase activity in the presence of $CaCl_2$.

Enzymatic activities	P_i liberated (μmol/mg protein per 30 min)
$(Na^+ + K^+)$-ATPase	
Control	6.84 ± 0.96
Ouabain, 0.1 mM	6.27 ± 0.84
Cyclic AMP, 50 μM	7.21 ± 0.69
Basal ATPase	
Control	6.24 ± 0.69
NaN_3, 5 mM	1.95 ± 0.30*
Cyclic AMP, 50 μM	6.70 ± 0.56
Ca^{2+}-activated ATPase, 100 μM $CaCl_2$	
Control	5.94 ± 0.60
NaN_3, 5 mM	6.09 ± 0.84
Cyclic AMP, 50 μM	6.28 ± 0.45
Ca^{2+}-activated ATPase, 10–25 μM $CaCl_2$	
Control	3.99 ± 0.43
NaN_3, 5 mM	3.87 ± 0.54
Cyclic AMP, 50 μM	6.08 ± 0.47*

Asterisks (*) indicate $P < 0.02$ for comparison of these values with the corresponding control values using the Student's t-test.

TABLE II

ENZYMATIC ACTIVITIES ASSOCIATED WITH SARCOPLASMIC RETICULUM

Enzymatic activities were determined in 2–6 preparations of sarcoplasmic reticulum. Adenylate cyclase was assayed [21] and expressed as nmol of cyclic AMP produced/mg protein per min. 3′ : 5′-cyclic nucleotide phosphodiesterase was estimated by measurement of the loss of cyclic AMP under conditions of the ATPase assay with 3–4 μM cyclic AMP and expressed as nmol of cyclic AMP hydrolyzed/mg protein per min. Cyclic AMP were determined by radioimmunoassay [21]. Cyclic AMP-stimulated protein kinase was measured as previously described [4] and expressed as nmol of ^{32}P precipitated/mg protein per min. Phosphoprotein phosphatase was assayed by measuring the dephosphorylation of ^{32}P-labeled, phosphorylated membranes obtained by centrifugation after phosphorylation for 30 min under the condition of the ATPase assay. The reaction mixture consisted of 0.2 ml with 0.1–0.2 mg of membrane protein/ml, 10 mM histidine, pH 7.0, 10 mM $MgCl_2$, 115 μM EGTA and 100 μM $CaCl_2$ with a Ca^{2+} concentration of 10.5 μM, calculated using an apparent dissociation constant for the calcium-EGTA complex of 3 μM [22, 23]. The reaction was terminated at 15 min by the addition of trichloroacetic acid as described in Methods. Phosphoprotein phosphatase was expressed as nmol of [^{32}P]phosphoprotein hydrolyzed/mg protein per min.

Enzymatic activities	nmol/mg protein per min
Adenylate cyclase	
Basal	0.04–0.08
(−)-Epinephrine, 10 μM	0.07–0.13
KF, 10 mM	0.25–0.55
Cyclic nucleotide phosphodiesterase	0.50–2.00
Cyclic AMP-stimulated protein kinase	0.08–0.15
Phosphoprotein phosphatase	0.01–0.02

assayed in the presence of EGTA has been cited as a marker of mitochondrial activity [18] and was present in these membranes, representing about two-thirds of the basal ATPase activity (Table I). This degree of azide sensitivity in basal ATPase has also been observed in other cardiac sarcoplasmic reticulum preparations [6, 18]. Azide did not effect Ca^{2+}-activated ATPase (Table I) or calcium uptake in the presence of oxalate as we noted previously [4], indicating that these activities were not related to mitochondrial contamination. Endogenous calcium in this sarcoplasmic reticulum was determined by atomic absorption spectrometry [19]. The total endogenous calcium in these membranes was 111 $\pm$9 nmol/mg protein (mean $\pm$S.E., $n = 6$) of which 60–75 % was extractable into the 100 000 $\times g$ supernatant by a 10 min incubation at 20 °C in the presence of 0.5 mM EGTA, in agreement with Entman et al. [17]. RNA contamination of these membranes was determined using the orcinol reaction after extraction into KOH [20] and was < 10 μg/mg of membrane protein. Catecholamine-sensitive adenylate cyclase, 3′ : 5′-cyclic nucleotide phosphodiesterase, cyclic AMP-stimulated protein kinase and phosphoprotein phosphatase were present in this preparation (Table II) and active to varying degrees under the conditions of the ATPase assay described below. Phosphoprotein phosphatase activity was high enough to be of possible importance in regulation of phosphoprotein during the ATPase assay but not high enough to contribute significantly to the formation of P_i.

Effect of cyclic AMP on ATPase activities

The effect of cyclic AMP on various ATPase activities was studied in four consecutive preparations of canine cardiac sarcoplasmic reticulum (Table I). Cyclic

AMP caused a statistically significant increase in Ca^{2+}-activated ATPase with 10–25 μM $CaCl_2$ (Table I) but had no effect on basal ATPase in the presence of EGTA or on Ca^{2+}-activated ATPase with 100 μM $CaCl_2$ (Table I). Cyclic AMP did not consistently increase Ca^{2+}-ATPase in the absence of magnesium (1.04 ± 0.66 and 1.24 ± 0.25, μmol/mg protein per 30 min, mean $\pm$S.E., $n = 4$). Cyclic AMP stimulation of ATPase activity required magnesium and low concentrations of $CaCl_2$, thus affecting only Ca^{2+}-activated ATPase which was determined by subtracting the basal ATPase activity in the presence of 0.5 mM EGTA from the ATPase activity in the presence of $CaCl_2$.

Cyclic AMP stimulation of Ca^{2+}-activated ATPase

Cyclic AMP stimulation of Ca^{2+}-activated ATPase was linear with time up to 40 min and was unaffected by membrane concentration (Figs. 1A and 1B). With maximal activation by 100 μM $CaCl_2$, Ca^{2+}-activated ATPase was linear for 40 min (Fig. 1A). Basal ATPase in the presence of 0.5 mM EGTA was also linear for 40 min (data not shown). The effects of cyclic AMP on Ca^{2+}-activated ATPase were routinely studied at 30 min with 10 μM $CaCl_2$.

The cyclic AMP stimulation of Ca^{2+}-activated ATPase was potentiated by inhibition of cyclic nucleotide phosphodiesterase. The cyclic AMP concentration required for half-maximal stimulation was reduced by 0.1 mM papaverine from approx. 3.0 to approx. 0.1 μM (Fig. 2). The membrane-associated phosphodiesterase was half-maximally inhibited by 20 μM papaverine when assayed under conditions of these experiments (see Table II). AMP at 1–100 μM did not stimulate Ca^{2+}-activated ATPase (Fig. 2). Cyclic GMP at 1–100 μM did not inhibit the stimulatory effect of

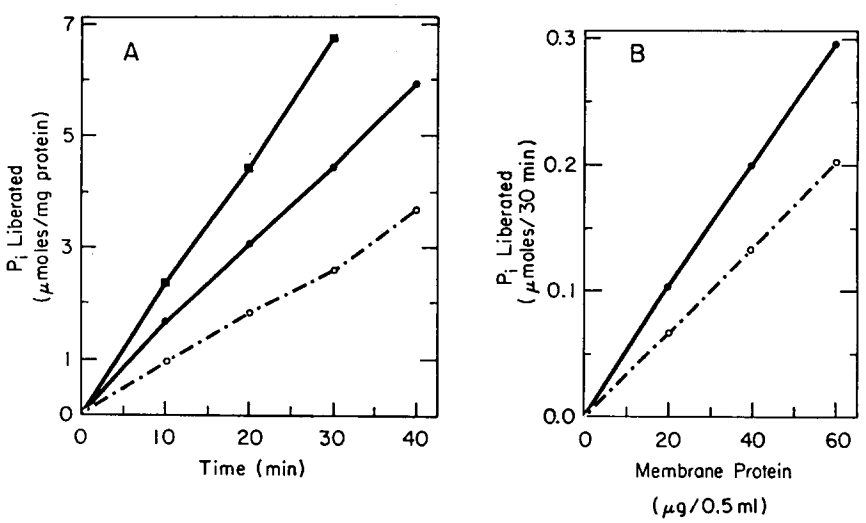

Fig. 1. (A) Time course of cyclic AMP stimulation of Ca^{2+}-activated ATPase. Ca^{2+}-activated ATPase with 10 μM $CaCl_2$ was determined at various times with (●) and without (○) 50 μM cyclic AMP as described in Methods. Ca^{2+}-activated ATPase with 100 μM $CaCl_2$ (■) was also determined. (B) Cyclic AMP stimulation of Ca^{2+}-activated ATPase at various membrane concentrations. Ca^{2+}-activated ATPase was determined at 30 min as described in A using another sarcoplasmic reticulum preparation.

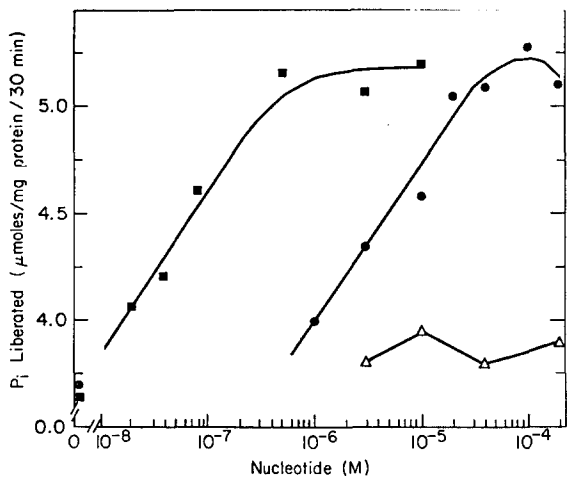

Fig. 2. Effect of various concentrations of cyclic AMP on Ca^{2+}-activated ATPase. Ca^{2+}-activated ATPase with 10 μM $CaCl_2$ was determined with various concentrations of cyclic AMP (●), AMP (△) and cyclic AMP plus 0.1 μM papaverine (■) as described in Methods. The endogenous cyclic AMP production in the presence of papaverine was measured by radioimmunoassay [21] and resulted in a final concentration of 0.018 μM cyclic AMP.

5 μM cyclic AMP and, at 100 μM, cyclic GMP alone stimulated Ca^{2+}-activated ATPase to the same extent as cyclic AMP.

Ca^{2+} requirement for cyclic AMP stimulation of Ca^{2+}-activated ATPase

The cyclic AMP stimulation of Ca^{2+}-activated ATPase required an optimal concentration of Ca^{2+} which was in the range of myoplasmic Ca^{2+} concentrations

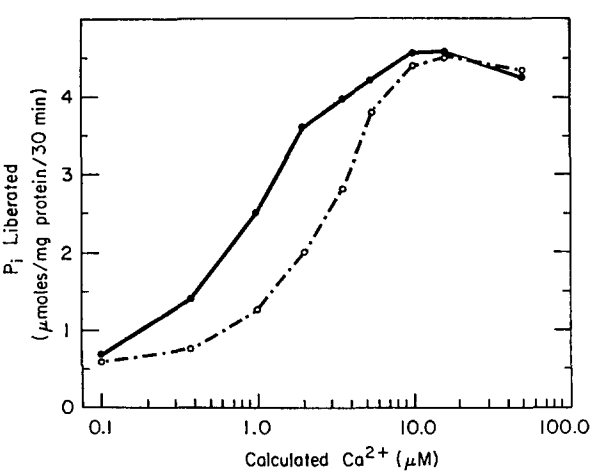

Fig. 3. Effect of Ca^{2+} concentrations on cyclic AMP stimulation of Ca^{2+}-activated ATPase. Ca^{2+}-activated ATPase was assayed with (●) and without (○) 40 μM cyclic AMP as described in Methods with 100 μM $CaCl_2$ and various concentrations of EGTA. The Ca^{2+} concentration was calculated using an apparent dissociation constant for the calcium-EGTA complex of 3 μM [22, 23].

[24] (Fig. 3). Assuming an apparent dissociation constant of 3 μM for the calcium-EGTA complex [22, 23], the cyclic AMP effect was maximal at a calculated Ca^{2+} of about 2 μM and insignificant at calculated [Ca^{2+}] of 0.1 and 10 μM. The Ca^{2+} required for half-maximal activation of Ca^{2+}-activated ATPase was reduced by greater than 50 % by cyclic AMP. In the routine ATPase assay with 10 μM $CaCl_2$ and no EGTA, the Ca^{2+} concentration was determined primarily by the calcium binding of ATP with a calculated [Ca^{2+}] of approx. 2.5 μM [18]. This calculated [Ca^{2+}] changed less than 25 % as a result of ATP hydrolysis and/or calcium binding by the sarcoplasmic reticulum reported by others [1, 17].

Cyclic AMP and calcium stimulation of membrane phosphorylation

Cyclic AMP increased membrane phosphorylation under the conditions required for cyclic AMP stimulation of Ca^{2+}-activated ATPase (Fig. 4A). Phosphorylation with and without cyclic AMP increased during the first 30 min. The cyclic AMP-dependent phosphorylation was maximal by 10 min and remained unchanged during the remainder of a 45 min reaction. When assayed at 30 min, cyclic GMP at 0.1–100 μM did not inhibit the stimulatory effect of 5 μM cyclic AMP and, at 100 μM, cyclic GMP alone stimulated membrane phosphorylation to about the same extent. Inclusion of 1 mM $K_2H^{32}PO_4$ as the only radioactive source in a reaction mixture with or without ATP resulted in < 3 % of the phosphorylation obtained in Fig. 4A at 10 and 30 min. In the presence of EGTA or 100 μM $CaCl_2$, cyclic AMP stimulation of membrane phosphorylation was markedly reduced (Fig. 4B). In both cases, phosphorylation with and without cyclic AMP increased during the first 30 min; whereas, the cyclic AMP-dependent phosphorylation was almost maximal at 10 min. In the absence of cyclic AMP, $CaCl_2$ concentration up to 100 μM stimulated membrane phosphorylation (Figs. 4A and 4B). Maximal calcium-dependent phosphorylation was obtained only after 30 min, indicating that it was not essential for earlier calcium activation of Ca^{2+}-activated ATPase (Fig. 1).

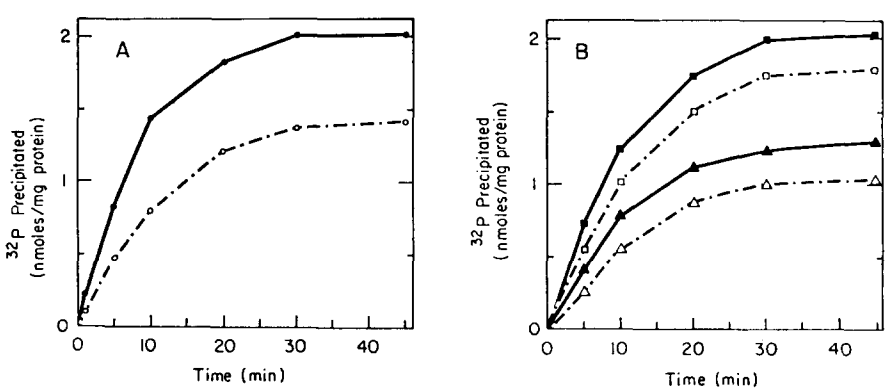

Fig. 4. Time course of cyclic AMP stimulation of membrane phosphorylation. Phosphorylation was determined at various times with (solid symbols) and without (open symbols) 50 μM cyclic AMP under the conditions of the ATPase assay with 10 μM $CaCl_2$ (●, ○) in A as well as with 0.5 mM EGTA (▲, △) or 100 μM $CaCl_2$ (■, □) in B (See Methods). Data represent the means of values from two sarcoplasmic reticulum preparations.

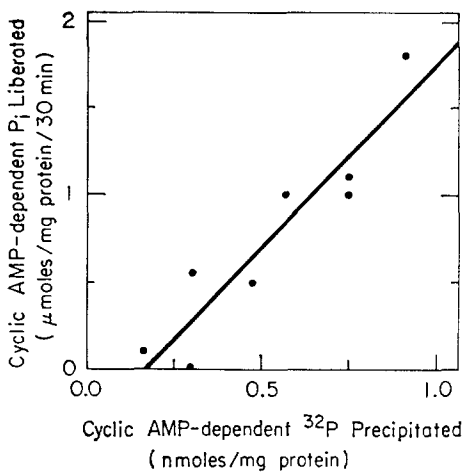

Fig. 5. Relationship of cyclic AMP-dependent Ca^{2+}-activated ATPase to cyclic AMP-dependent membrane phosphorylation at various $CaCl_2$ concentrations. Ca^{2+}-activated ATPase was assayed as described in Methods with and without 50 μM cyclic AMP in the presence of 0.5 mM EGTA or of $CaCl_2$ from 1 to 1000 μM. Membrane phosphorylation at 30 min was determined as described in Methods under the same conditions as the Ca^{2+}-activated ATPase. The cyclic AMP-dependent values were obtained under each condition by subtracting the value without cyclic AMP from the value with cyclic AMP. Data represent the means of two experiments. The line shown was determined by least squares linear regression analysis ($r = 0.93$, $P < 0.01$).

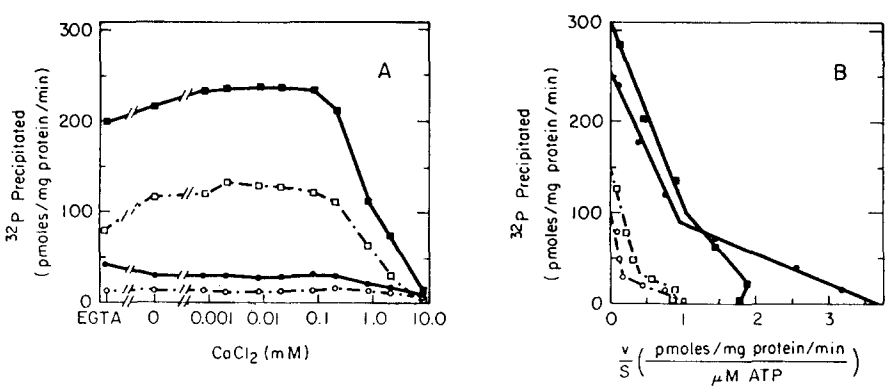

Fig. 6. Effect of calcium on endogenous protein kinase activity. Protein kinase activity was assayed with (solid symbols) and without (open symbols) 25 μM cyclic AMP as described in Methods using various concentrations of ATP and various concentrations of $CaCl_2$ or 0.5 mM EGTA. The data represent the means from two to three sarcoplasmic reticulum preparations. Individual experiments were performed at one ATP concentration with various concentrations of $CaCl_2$ or EGTA. (A) Protein kinase activity was determined as noted above with 16 μM (●, ○) and 1.6 mM (■, □) ATP. (B) Hofstee plots (v vs. v/S) for protein kinase activity at various ATP concentrations were constructed for kinase in the presence and in the absence of cyclic AMP with 0.5 mM EGTA (●, ○) or 10 μM $CaCl_2$ (■, □). The slopes and Y-intercepts of the lines were calculated using least squares linear regression analysis using data from ATP concentrations of 5–50 μM or 160–1600 μM. This interpolation of data tended to increase the K_m and V of the low K_m kinase activity and to decrease the K_m and V of the high K_m kinase activity.

Cyclic AMP stimulation of both Ca^{2+}-activated ATPase and membrane phosphorylation required an optimal calcium concentration. A linear correlation ($r = 0.93$) was demonstrated between cyclic AMP-dependent Ca^{2+}-activated ATPase and cyclic AMP-dependent membrane phosphorylation when determined with $CaCl_2$ concentrations of 1–1000 μM and with 0.5 mM EGTA (Fig. 5).

Effect of calcium on endogenous protein kinase activity

When membrane phosphorylation was studied at 1 min, the effect of $CaCl_2$ on endogenous protein kinase activity was dependent on the ATP concentration (Fig. 6A). At high ATP concentrations, $CaCl_2$ at 5–100 μM increased both cyclic AMP-independent protein kinase and cyclic AMP-stimulated protein kinase activities by 40–50 pmol/mg protein per min above their rates in the presence of 0.5 mM EGTA. 1 mM $CaCl_2$ decreased both activities to 50 % of their levels with 10 μM $CaCl_2$. Cyclic AMP-dependent protein kinase activity, however, was not increased by $CaCl_2$ up to 100 μM and decreased by 65 % at 1 mM $CaCl_2$ (Fig. 6A). At low ATP concentrations, the omission of EGTA resulted in a 30 % decrease in cyclic AMP-stimulated protein kinase activity which was decreased to 50 % at 1 mM $CaCl_2$ (Fig. 6A). Cyclic AMP-dependent protein kinase activity had a similar pattern of inhibition. Cyclic AMP-independent protein kinase activity was not inhibited until $CaCl_2$ reached 1 mM (Fig. 6A). These data suggested the presence of a calcium-stimulated protein kinase similar to phosphorylase *b* kinase [25–27] in addition to the cyclic AMP-stimulated protein kinase we demonstrated previously [4].

The apparent K_m values for ATP and the V values of protein kinase activities were estimated by a computer program for two K_m enzyme reactions [28] and by Hofstee plots [29]. Computer analysis of data obtained in the presence of EGTA and cyclic AMP estimated K_m values of 15 and 320 μM (Table III). The approximations of K_m from the Hofstee plots of data obtained in the presence of EGTA (Fig. 6B) were comparable to the K_m values of the computer analysis (Table III). Hofstee plots of

TABLE III

ESTIMATES OF APPARENT K_m AND V OF PROTEIN KINASE ACTIVITIES

Protein kinase activities were determined as described in Fig. 6. A computer program for two K_m enzyme reactions [28] analyzed data obtained using eleven ATP concentrations (5–1600 μM) in the presence of EGTA and cyclic AMP. The K_m and V values were expressed as estimates ±asymptotic S.D. The K_m and V values calculated by the Hofstee plots in Fig. 6B were expressed as the estimates ±S.E.

Type of analysis and assay conditions	K_{m1} (μM)	K_{m2} (μM)	^{32}P precipitated (pmol/mg protein per min)	
			V_1	V_2
Computer				
EGTA and cyclic AMP	15±9	320± 91	75±21	265±30
Hofstee				
EGTA	24±5	310±107	32± 4	92±7
EGTA and cyclic AMP	30±4	220± 60	120±13	260±13
$CaCl_2$	–	304±125	–	146±14
$CaCl_2$ and cyclic AMP	–	206± 86	–	310±30

data obtained in the presence of $CaCl_2$ were non-linear at low ATP concentrations but allowed approximations at high ATP concentrations (Fig. 6B). The high K_m values for ATP were similar with and without $CaCl_2$ (Table III). The V of calcium-inhibited kinase activity with a low K_m for ATP was stimulated from 32 to 120 pmol/mg protein per min by cyclic AMP in the presence of EGTA (Table III). The V of the calcium-stimulated kinase activity with the high K_m for ATP was increased approx. 165 pmol/mg protein per min by cyclic AMP in the presence of either EGTA or 10 μM $CaCl_2$ (Table III). Cyclic AMP at 0.08 μM produced half-maximal stimulation of kinase activity with 10 μM $CaCl_2$ and 0.5 mM ATP. The inability to completely inhibit the high K_m kinase with EGTA may have been related to the ineffectiveness of the EGTA in extraction of membrane calcium (see Methods).

Characterization of phosphorylated membranes

Sarcoplasmic reticulum phosphorylated under conditions of either the endogenous protein kinase assay or the ATPase assay had similar characteristics (Table IV). ^{32}P in trichloroacetic acid precipitates was resistant to extraction by hot trichloro-

TABLE IV

CHARACTERIZATION OF PHOSPHORYLATED MEMBRANES

Phosphorylated membranes were prepared under conditions of the endogenous protein kinase assay with 25 μM cyclic AMP and 20 μM ATP (No. 1) or of the ATPase assay with 10 μM $CaCl_2$ and 25 μM cyclic AMP (No. 2) or with 100 μM $CaCl_2$ (No. 3). Phosphorylated membranes were treated as described below and expressed as a percent of control. (See Methods for details).

Treatments of phosphorylated membranes	Percent recovery of ^{32}P-labeled, phosphorylated membranes		
	No. 1	No. 2	No. 3
(A) As trichloroacetic acid precipitates			
(1) 10 % trichloroacetic acid at 0 °C for 15 min	100	100	100
(2) 10 % trichloroacetic acid at 90 °C for 15 min	88	87	77
(3) ethanol/ethyl ether/chloroform (2 : 2 : 1, v/v) 25 °C for 15 min	87	102	105
(4) 150 mM sodium β-glycerophosphate, pH 7.0, at 90 °C for 15 min	85	83	72
(5) 1 M NaOH at 90 °C for 15 min	0	3	8
(6) 0.8 M NaCl, 0.1 M sodium acetate, pH 5.5, at 30 °C for 15 min	82	85	85
(7) 0.8 M hydroxylamine, 0.1 M sodium acetate, pH 5.5 at 30 °C for 15 min	77	89	82
(B) As 180 000 $\times g$ pellets			
(1) 50 mM sodium β-glycerophosphate, pH 7.0, at 0 °C for 30 min	100	–	–
(2) 1 mg/ml Triton X-100 at 0 °C for 30 min	97	–	–
(3) 50 mM sodium β-glycerophosphate, pH 7.0, at 25 °C for 30 min twice	100	100	–
(4) 0.6 M KCl, 50 mM sodium β-glycerophosphate, pH 7.0, at 25 °C for 30 min twice	85	91	–

acetic acid or by ethanol/ethyl ether/chloroform, ruling out nucleic acid or lipid as the major phosphorylated component. This ^{32}P was readily hydrolyzed by hot 1 M NaOH but not by 0.8 M hydroxylamine, indicating the presence of phosphoester bonds [30]. ^{32}P in phosphorylated membrane pellets was not extracted by 1 mg/ml Triton X-100, which readily extracts over 90 % of the cyclic AMP-dependent protein kinase in these membranes [4]. Over 85 % of the membrane ^{32}P was recovered after two washings of the membrane pellets with buffered 0.6 M KCl, indicating that the phosphoprotein was mainly membrane associated rather than contractile protein [16, 31] which can be similarly phosphorylated [32–34].

SDS discontinuous polyacrylamide gel electrophoresis of microsomal proteins

Sarcoplasmic reticulum solubilized in 2 % SDS revealed a distinct pattern of protein staining after SDS discontinuous polyacrylamide gel electrophoresis (Fig. 7). The great majority of the protein staining was observed in the 30 000–100 000 molecular weight range with the largest staining peak between 90 000 and 100 000. This 90 000–100 000 dalton peak has been noted in cardiac sarcoplasmic reticulum [16] and shown to contain the acyl phosphoprotein intermediate of ATPase [35]. Relative-

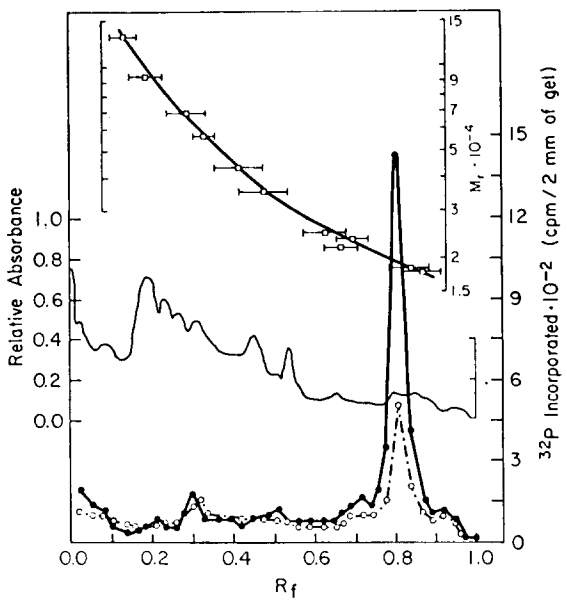

Fig. 7. Effect of cyclic AMP on the gel electrophoretic patterns of phosphorylated membranes. Sarcoplasmic reticulum was phosphorylated for 30 min as described in Methods with 10 μM $CaCl_2$ in the presence (●) or absence (○) of 25 μM cyclic AMP. After solubilization in 2 % SDS, 90 μg of membrane protein was applied to the SDS discontinuous polyacrylamide gel electrophoresis system. After electrophoresis and staining for protein, the relative absorbance of the gel and ^{32}P per 2 mm of gel were determined (see Methods). The mean R_F values (□) for the protein standards with S.D. represented by brackets were determined during these experiments (see Methods). Protein standards (molecular weight, M_r) were sperm whale myoglobin (17 800), β-lactoglobulin (18 400), human γ-globulin light chain (22 500), trypsin (23 300), chymotrypsinogen (25 700), pepsin (35 000), ovalbumin (43 000), human γ-globulin heavy chain (57 500), bovine serum albumin (68 000), phosphorylase *a* (94 000) and β-galactosidase (130 000).

ly little protein staining was noted below M_r 30 000; however, a consistent peak was found approx. at M_r 20 000. This peak represented between 2 and 5 % of the total stainable protein when estimated by the absorbance in the gels. Phosphorylation with and without cyclic AMP under the conditions of the ATPase assay did not change the pattern of protein staining in the SDS gels.

Phosphorylation of different molecular weight membrane proteins

Phosphorylated membrane components were localized in three molecular weight peaks by SDS discontinuous polyacrylamide gel electrophoresis. Cyclic AMP caused a 2–3-fold increase of ^{32}P incorporation in the 20 000 dalton peak (M_r = 20 000±1500, mean ±S.E., n = 12) under conditions of the ATPase assay which yielded maximal cyclic AMP-dependent membrane phosphorylation (Fig. 7). Smaller ^{32}P peaks at M_r = 55 000 and at M_r > 130 000 were consistently observed (Fig. 7). In eight sarcoplasmic reticulum preparations incubated for 30 min, cyclic AMP significantly stimulated ^{32}P incorporation in the 20 000 dalton peak from 0.21±0.03 to 0.51±0.09 (mean ±S.E., ^{32}P incorporated, pmol/μg of total protein applied to gel) but had no consistent effect on ^{32}P incorporation in the other peaks (M_r 55 000, 0.20±

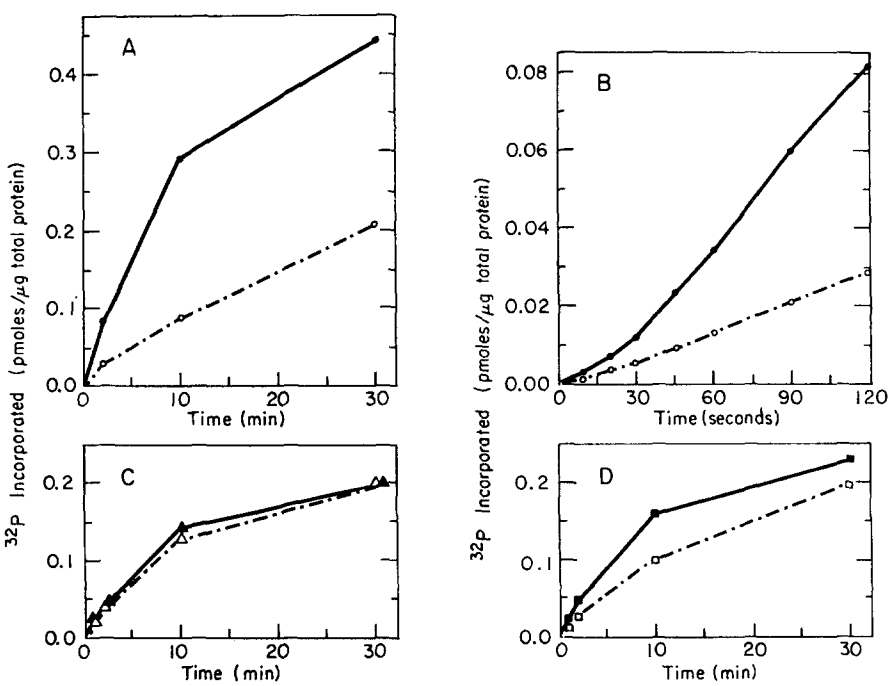

Fig. 8. Time course of the effect of cyclic AMP on phosphorylation of different molecular weight membrane proteins. Sarcoplasmic reticulum was phosphorylated for various times in the presence of 10 μM $CaCl_2$ with (solid symbols) and without (open symbols) cyclic AMP as in Fig. 7. ^{32}P incorporation in the 20 000 dalton peak (●, ○) in A and B, the 55 000 dalton peak (▲, △) in C and the > 130 000 dalton peak (■, □) in D was determined as in Fig. 7. ^{32}P incorporation was expressed in terms of the total protein applied to the gel. Data represent means from two experiments. The amount of ^{32}P recovered in these gels represented about 70 % of the total ^{32}P applied to the gels.

0.05 to 0.20±0.05 and M_r > 130 000, 0.18±0.04 to 0.23±0.07). The amount of ^{32}P recovered in these three peaks represented about 65 % of the total ^{32}P in the gels. Cyclic GMP at 0.001–0.1 μM did not inhibit the cyclic AMP (25 μM) stimulation of phosphorylation in the 20 000 dalton peak and, at 10 μM, cyclic GMP alone stimulated phosphorylation in the 20 000 dalton peak about 2-fold.

The phosphorylation of each of these three peaks with and without cyclic AMP increased during the first 30 min (Fig. 8). The cyclic AMP-dependent phosphorylation of the 20 000 dalton peak was maximal at 10 min and remained unchanged thereafter (Fig. 8A), similar to the pattern noted for the cyclic AMP-dependent membrane phosphorylation (Fig. 4). The rate of cyclic AMP-dependent phosphorylation of this peak increased markedly during the first 30 s, remained constant from 30 to 120 s then declined (Figs. 8A and 8B). The initial rates of phosphorylation of the other two peaks were constant during the first 2 min then declined (Figs. 8C and 8D). The phosphorylation of the 55 000 dalton peak was not increased by cyclic AMP: whereas, the phosphorylation of > 130 000 dalton peak was stimulated 2-fold by cyclic AMP during the first 2 min.

Effect of calcium on phosphorylation of 20 000 molecular weight membrane protein

$CaCl_2$ from 10 to 100 μM increased ^{32}P incorporation in the 20 000 dalton peak in the presence and absence of cyclic AMP (Fig. 9). $CaCl_2$ at 1 mM or greater inhibited the ^{32}P incorporation. The cyclic AMP-dependent phosphorylation of 20 000 molecular weight membrane protein was maximal at 10 μM $CaCl_2$ and represented a

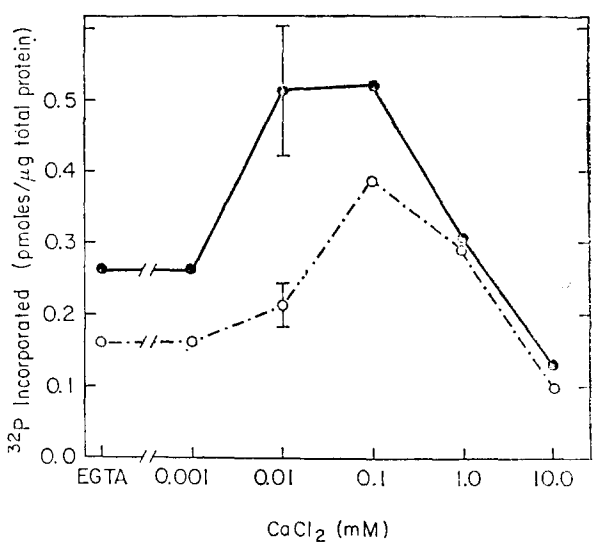

Fig. 9. Effect of calcium on phosphorylation of 20 000 molecular weight membrane protein. Membranes were incubated for 30 min as in Fig. 8 in the presence (●) or absence (○) of 25 μM cyclic AMP with varying concentrations of $CaCl_2$ or with 0.5 mM EGTA. ^{32}P incorporation in the 20 000 dalton peak was determined and expressed as described in Fig. 8. The data represent the means obtained from two sarcoplasmic reticulum preparations except at 0.01 mM $CaCl_2$ which are the means ±S.E. obtained from eight preparations. At 0.01 mM $CaCl_2$ the effect of cyclic AMP was significant with a $P < 0.02$ using the Student's *t*-test.

2–3-fold increase over the phosphorylation without cyclic AMP. Cyclic AMP-dependent phosphorylation was completely inhibited at 1 mM $CaCl_2$ and was decreased to 33 % at 1 μM $CaCl_2$. $CaCl_2$ had similar effects on cyclic AMP-dependent membrane phosphorylation (Figs. 4 and 5).

Effect of trypsin treatment on microsomal activities

In trypsin-treated microsomes cyclic AMP did not stimulate Ca^{2+}-activated ATPase or phosphorylation of the 20 000 molecular weight membrane protein. When assayed as described in Fig. 1 with 10 μM $CaCl_2$, cyclic AMP (25 μM) had no effect on Ca^{2+}-activated ATPase (3.06 ± 0.33 and 3.26 ± 0.41, μmol/mg protein per 30 min, mean $\pm$S.E., $n = 3$). Under the same conditions as described in Fig. 7, phosphorylation of the 20 000 molecular weight protein was decreased to < 0.03 pmol of ^{32}P incorporated/μg of total protein applied to the gel while the phosphorylation of other peaks was decreased by only 12–20 %.

DISCUSSION

Cardiac sarcoplasmic reticulum has proven difficult to isolate without contamination by other membrane components [18]. Although sarcolemmal enzymatic activities and RNA content were low in our preparation, mitochondrial contamination as evidenced by azide-sensitive basal ATPase was present in this as in other preparations of cardiac sarcoplasmic reticulum [10, 18]. Nonetheless, the membrane vesicles used in these studies were characteristic of fragmented sarcoplasmic reticulum in that they maintained high levels of Ca^{2+}-activated ATPase and azide-insensitive calcium uptake in the presence of oxalate [10, 17, 36]. In addition this sarcoplasmic reticulum contained a complete enzymatic system for regulating cyclic AMP-mediated effects, having an epinephrine-sensitive adenylate cyclase, cyclic nucleotide phosphodiesterase, cyclic AMP-stimulated protein kinase, protein substrate and phosphoprotein phosphatase. Excepting cyclic nucleotide phosphodiesterase, the components of this system have been demonstrated in other preparations of cardiac sarcoplasmic reticulum [2, 3, 16, 36–40].

The cyclic AMP stimulation of Ca^{2+}-activated ATPase in this cardiac sarcoplasmic reticulum appeared to require the cyclic AMP-dependent phosphorylation of 20 000 molecular weight membrane protein as evidenced by the ineffectiveness of cyclic AMP on trypsin-treated microsomes and by the correlation between cyclic AMP-dependent membrane phosphorylation and cyclic AMP-dependent Ca^{2+}-activated ATPase over a wide range of calcium concentrations. Kirchberger et al. [3] demonstrated a similar correlation between membrane phosphoprotein dependent on various amounts of exogenous protein kinase in the presence of cyclic AMP and subsequent oxalate-dependent calcium uptake by cardiac sarcoplasmic reticulum. In the studies reported here, approx. 80 % of cyclic AMP-dependent membrane phosphorylation occurred in 20 000 molecular weight membrane protein. The endogenous cyclic AMP-dependent membrane phosphorylation reported by La Raia and Morkin [2] was limited to a peak of approx. 20 000 daltons. Tada et al. [16] found that the membrane components phosphorylated by exogenous protein kinase in the presence of cyclic AMP were mainly in a 22 000 dalton peak with two small peaks at $M_r = 55\,000$ and 15 000 noted only in the presence of NaF.

The phosphorylation of 20 000 molecular weight membrane protein was mediated by a calcium-stimulated protein kinase which resembles skeletal muscle phosphorylase *b* kinase in terms of ATP and calcium requirements [25–27]. Phosphorylase *b* kinase has been demonstrated in cardiac and skeletal sarcoplasmic reticulum where it was also incompletely inhibited by EGTA [41, 42]. Phosphorylation of the $M_r = 20\,000$ component was calcium dependent and involved phosphoester bonds which are characteristic of the substrates of cyclic AMP-stimulated protein kinase as well as phosphorylase *b* kinase [43–46]. The cyclic AMP-stimulated phosphorylation of 20 000 molecular weight protein was increased during the first 30 s which is consistent with phosphorylation by calcium-stimulated protein kinase which is being activated by cyclic AMP-stimulated protein kinase. Further evidence of the role of calcium-stimulated protein kinase in regulating cardiac sarcoplasmic reticulum function was reported by Entman et al. [42, 47]. Exogenous phosphorylase *b* kinase increased both oxalate-dependent calcium uptake as well as Ca^{2+}-activated ATPase.

Cyclic AMP stimulation of the initial phosphorylation of $M_r > 130\,000$ membrane protein may represent the phosphorylation and activation of the catalytic subunit of phosphorylase *b* kinase by cyclic AMP-stimulated protein kinase [48, 49]. The same concentration of cyclic AMP was required for half-maximal stimulation of Ca^{2+}-activated ATPase, membrane phosphorylation and cyclic AMP-stimulated protein kinase [4]. This implicated the latter in the phosphorylation necessary for activation of Ca^{2+}-activated ATPase; however, cyclic AMP-stimulated protein kinase seemed not to phosphorylate 20 000 molecular weight protein directly. Cyclic AMP-stimulated protein kinase was inhibited by calcium; whereas, the phosphorylation of the membrane protein was stimulated by calcium. The phosphoprotein of $M_r = 55\,000$ which was unchanged by cyclic AMP may represesent the phosphorylated cyclic AMP-binding subunit of cyclic AMP-stimulated protein kinase [16, 50].

Cyclic GMP did not inhibit the cyclic AMP stimulation of Ca^{2+}-activated ATPase indicating that the negative inotropic response attributed to cyclic GMP is not mediated by antagonism of this cyclic AMP effect [51].

The studies in this paper demonstrate the cyclic AMP stimulation of Ca^{2+}-activated ATPase in cardiac sarcoplasmic reticulum. This stimulation is calcium dependent and is correlated with cyclic AMP-dependent membrane phosphorylation of which the great majority is a 20 000 molecular weight protein. The cyclic AMP stimulation of membrane phosphorylation involves two protein kinases and suggests that an activated (phosphorylated) calcium-stimulated protein kinase mediates the phosphorylation of the 20 000 molecular weight protein. These data support the hypothesis that some of the effects of cyclic AMP on the regulation of intracellular calcium concentrations in myocardium are mediated by a series of reactions similar to those activating phosphorylase *b* [41].

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