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MEMBRANE LOCALIZATION OF MYOCARDIAL TYPE II CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY

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Summary

Crude cardiac membrane vesicles were separated into subfractions of sarcolemma and sarcoplasmic reticulum. The subfractions were used to determine the origin and type of cyclic AMP-dependent protein kinase activity present in myocardial membranes. A cyclic AMP-binding protein of molecular weight 55 000 was covalently labeled with the photoaffinity probe 8-azido adenosine 3′,5′-mono[^{32}P]phosphate, and found to copurify with the ($Na^+ + K^+$)-ATPase activity of sarcolemma, and away from the ($Ca^{2+} + K^+$)-ATPase activity of sarcoplasmic reticulum. Endogenous cyclic AMP-dependent protein kinase activity also copurified with sarcolemma. Protein substrates phosphorylated by cyclic AMP-dependent protein kinase activity had apparent molecular weights of 21 000 and 8000 and were present in both sarcolemma and sarcoplasmic reticulum. However, while addition of cyclic AMP alone resulted in phosphorylation of sarcolemma proteins, both cyclic AMP and exogenous, soluble cyclic AMP-dependent kinase were required for phosphorylation of sarcoplasmic reticulum proteins. Addition of the calcium-binding protein, calmodulin, to either sarcolemma or sarcoplasmic reticulum resulted in phosphorylation of the 21 000 and 8000-dalton proteins, as well. The results suggest that cardiac sarcolemma contains an intrinsic type II cyclic AMP-dependent protein kinase activity that is not present in sarcoplasmic reticulum. On the other hand, Ca^{2+}- and calmodulin-dependent protein kinase activity is present in both sarcolemma and sarcoplasmic reticulum.

Abbreviations: R_{II}, regulatory subunit of type II cyclic AMP-dependent protein kinase; 8-N_3-[^{32}P]cAMP, 8-azido adenosine 3′,5′-mono[^{32}P]phosphate; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate.

Introduction

The intracellular effects of cyclic AMP in eukaryotic cells are most probably mediated by the activation of cyclic AMP-dependent protein kinases [1,2]. In various tissues both soluble and membrane-bound cyclic AMP-dependent protein kinase activities have been identified, which are broadly categorized into two main types, I and II. The two protein kinase activities have been differentiated by the properties of their regulatory subunits, whereas their catalytic subunits appear to be quite similar [3]. The distinguishing features of the type II cyclic AMP-dependent protein kinase activity include a regulatory subunit which migrates with an apparent molecular weight of 54 000—56 000, a regulatory subunit which is autophosphorylated by its associated catalytic subunit, and a holoenzyme which is eluted by high salt concentrations from DEAE-cellulose columns [4]. The membrane bound cyclic AMP-dependent protein kinase activity of neuronal plasma membranes is though to be predominantly type II [5,6], whereas for red blood cell membranes the cyclic AMP-dependent protein kinase activity was recently shown to be type I [7].

Although much work has been performed on soluble cyclic AMP-dependent protein kinase activities from cardiac tissue [8], the membrane bound activities are less well characterized [9]. Nonetheless, cyclic AMP-mediated phosphorylations of both sarcolemma and sarcoplasmic reticulum proteins have been implicated in the regulation of cardiac contractility [9]. In the present study we have used relatively purified preparations of sarcolemma and sarcoplasmic reticulum to determine the membrane origin and type of cyclic AMP-dependent protein kinase activity that is present in canine myocardium. Membrane purity was documented by assay of $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + K^+)$-ATPase activities of the sarcolemma and sarcoplasmic reticulum fractions, and by direct visualization of the acylphosphoprotein intermediates of either enzyme with autoradiography. Using a cyclic AMP photoaffinity probe which attaches covalently to proteins upon photolysis, we have labeled a cyclic AMP-binding protein which copurifies with the $(Na^+ + K^+)$-ATPase-specific activity of cardiac membranes. Furthermore, addition of cyclic AMP in the presence of ATP results in the phosphorylation of sarcolemma proteins, whereas phosphorylation of sarcoplasmic reticulum proteins requires both cyclic AMP and exogenous protein kinase. For comparative purposes we have also measured the Ca^{2+}-and calmodulin-dependent protein kinase activity of the membrane fractions [10]. Our results suggest that the membrane bound cyclic AMP-dependent protein kinase of canine myocardium is predominantly a type II enzyme localized to the sarcolemma, while the Ca^{2+} and calmodulin-dependent protein kinase is present in both sarcolemma and sarcoplasmic reticulum.

Experimental procedures

Isolation of sarcolemma and sarcoplasmic reticulum fractions. Sarcolemma and sarcoplasmic reticulum fractions were isolated by a procedure slightly modified from our previous report [11], to be published in detail elsewhere. Briefly, crude canine cardiac microsomes enriched in either sarcolemma vesicles or sarcoplasmic reticulum vesicles were subjected to further purifica-

tion by sucrose density gradient centrifugation [11]. Vesicles were preloaded with calcium oxalate to increase selectively the density of sarcoplasmic reticulum vesicles and thus facilitate a better separation of sarcolemma from sarcoplasmic reticulum on sucrose gradients. The sarcolemma fraction was collected at the interface of 0.6 M sucrose. Two sarcoplasmic reticulum fractions were collected, one at the interface of 1.5 M sucrose (fraction D), and the other which pelleted through 1.5 M sucrose (fraction E) [11]. Media of similar ionic strength and composition were used for purification of sarcolemma and sarcoplasmic reticulum, and a high ionic strength salt extraction step was used in both procedures (0.60—0.75 M KCl or NaCl) [11]. Membrane vesicles were pretreated with the channel-forming ionophore alamethicin at an alamethicin-to-protein ratio of 1 (w/w) for all assays described below, except for the assay in which Ca^{2+}- and calmodulin-dependent protein kinase activity was measured (Fig. 4). Alamethicin was required to eliminate permeability barriers in the vesicles, so that total enzymic specific activties could be measured [12,13]. Protein concentrations were determined by the method of Lowry et al. [14].

Assay of $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + K^+)$-ATPase activities. $(Na^+ + K^+)$-ATPase activity was determined at 37°C in a medium containing 50 mM histidine (pH 7.0), 3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 1 mM EGTA, 3 mM phospho*enol*pyruvate, and 50 μg/ml pyruvate kinase. $(Na^+ + K^+)$-ATPase activity was that activity inhibited by 1 mM ouabain. $(Ca^{2+} + K^+)$-ATPase activity was determined at 37°C in a medium containing 50 mM histidine (pH 7.0), 3 mM $MgCl_2$, 100 mM KCl, 25 μM $CaCl_2$, 3 mM phospho*enol*pyruvate, and 50 μg/ml pyruvate kinase. $(Ca^{2+} + K^+)$-ATPase activity was that activity inhibited by 1 mM EGTA. The ATP concentration used for both assays was 3 mM. Inorganic phosphate released from ATP was determined colorimetrically [13]. [γ-^{32}P]ATP-phosphorylated acylphosphoprotein intermediates of $(Na^+ + K^+)$-ATPase and $(Ca^{2+} + K^+)$-ATPase were visualized by autoradiography after electrophoresis of the phosphorylated membranes by the procedure of Fairbanks et al. [15], as recently described [16].

Assay of protein kinase activities. For assay of cyclic AMP-dependent protein kinase activity membrane vesicles were preincubated for 5 min at 30°C in 40 μl of a medium containing 0.12 M sucrose, 50 mM phosphoric acid, pH 6.8 (by addition of Tris base), 10 mM $MgCl_2$, 1 mM Tris-EGTA, and 1 mM 3-isobutyl-1-methylxanthine, with or without 10 μM cyclic AMP. For assay of Ca^{2+}- and calmodulin-dependent protein kinase activity, membrane vesicles were preincubated for 5 min at 30°C in 40 μl of a medium containing 0.12 M sucrose, 25 mM phosphoric acid (pH 6.8), 12.5 mM $MgCl_2$, and 10 mM Tris-EGTA with or without 8 mM $CaCl_2$ and $1 \cdot 10^{-5}$ M calmodulin. Calmodulin was purified from bovine testicle by the method of Klee [17]. Phosphorylation was initiated by adding 10 μl of [γ-^{32}P]ATP ($6 \cdot 10^6$ dpm) to a final concentration of 10 μM for assay of both protein kinase activities. After a 1 min incubation at 30°C, reactions were terminated by adding 20 μl of 10% SDS, 40 mM dithiothreitol, 62.5 mM Tris base (pH 6.8) and 10% glycerol. Electrophoresis of phosphorylated proteins was slightly modified [13] from the procedure of Porzio and Pearson [18]. The polyacrylamide gels were dried and autoradiography was done with

Dupont Cronex-4 medical X-ray film using Quanta III intensifying screens [19]. The gels were stained with Coomassie blue prior to drying, and the protein standards used for estimation of molecular weights were myosin (200 000), RNA polymerase subunits (165 000; 155 000; 39 000), phosphorylase *a* (92 000), bovine serum albumin (68 000), ovalbumin (45 000), trypsinogen (24 000), γ-lactoglobulin (18 400), lysozyme (14 300), and aprotinin (6500).

Photoaffinity labeling with 8-N_3-[^{32}P]cAMP. Photoaffinity labeling was slightly modified from the method of Walter et al. [5]. Membrane vesicles were preincubated for 15 min at room temperature in the dark in the same buffer solution used for assay of cyclic AMP-dependent protein kinase activity, with the additional presence of 0.2 μM 8-N_3-[^{32}P]cAMP with or without 20 μM non-radioactive cyclic AMP. The samples were then irradiated at 4°C for 10 min at 254 nm with a Mineralite hand lamp at a distance of 6 cm. Membranes were processed for autoradiography as above after adding the SDS solution to stop the reactions.

Materials. [γ-^{32}P]ATP and 8-N_3-[^{32}P]cAMP were purchased from ICN Pharmaceuticals. Soluble type II cyclic AMP-dependent protein kinase from beef heart was obtained from the Sigma Chemical Co.

Results

(Na^+ + K^+)-ATPase and (Ca^{2+} + K^+)-ATPase activities were measured in the three membrane fractions sarcolemma, fraction D, and fraction E to verify their relative purities (Table I). All activities were determined in the presence of the peptide ionophore alamethicin, which obviates any enzymic latency exhibited by the sealed membrane vesicles [12,13]. The two sarcoplasmic reticulum fractions (D and E) were studied concomitantly, because preliminary

TABLE I

ATPase ACTIVITIES AND 8-N_3-[^{32}P]cAMP PHOTOAFFINITY LABELING OF SARCOLEMMA AND SARCOPLASMIC RETICULUM FRACTIONS

Incorporation of 8-N_3-[^{32}P]cAMP into membrane fractions was determined by cutting the R_{II} regions from the polyacrylamide gel of Fig. 2 and quantitating radioactivity with a scintillation counter. Sl, sarcolemma; SR, sarcoplasmic reticulum; cAMP, cyclic AMP.

Activity measured		Membrane fraction		
		Sl	SR_D	SR_E
(Na^+ + K^+)-ATPase (μmol P_i/mg protein per h)	(—) Ouabain	191	31.2	21.2
	(+) Ouabain	20.3	19.6	14.1
	Difference	171	11.6	7.1
(Ca^{2+} + K^+)-ATPase (μmol P_i/mg protein per h)	(—) EGTA	24.7	102	191
	(+) EGTA	20.6	22.4	17.8
	Difference	4.1	79.6	173
8-N_3-[^{32}P]cAMP incorporation (cpm/15 μg protein)	(—) cAMP	648	68	40
	(+) cAMP	100	26	18
	Difference	548	42	22

data suggest that they may be functionally different [20]. The ouabain sensitive ($Na^+ + K^+$)-ATPase activity of the three fractions was highest in sarcolemma and lowest in the two sarcoplasmic reticulum fractions, which had 7% or less of the sarcolemma activity (Table I), The ($Ca^{2+} + K^+$)-ATPase activity copurified with the two sarcoplasmic reticulum fractions, and away from the sarcolemma fraction. The ($Ca^{2+} + K^+$)-ATPase activity of the sarcolemma fraction was 5% or less of that of the two sarcoplasmic reticulum fractions. The validity of using ($Na^+ + K^+$)-ATPase as an enzymic marker for sarcolemma and ($Ca^{2+} + K^+$)-ATPase as an enzymic marker for sarcoplasmic reticulum in myocardium has been documented by us previously [11,16,21] and also by Misselwitz et al. [22] in an independent study.

The acylphosphoprotein intermediates of the ($Na^+ + K^+$)-ATPase and ($Ca^{2+} + K^+$)-ATPase activities of sarcolemma, and of D and E fractions were visualized by autoradiography (Fig. 1). Na^+-dependent phosphorylation of a protein of molecular weight 100 000 was evident in only the sarcolemma fraction (Fig. 1, lane 1), whereas Ca^{2+}-dependent phosphorylation of proteins of similar mobility was evident in only the two sarcoplasmic reticulum fractions (Fig. 1, lanes 9 and 11). Since Na^+-dependent phosphorylation specifically labels the ($Na^+ + K^+$)-ATPase of sarcolemma [16], and Ca^{2+}-dependent phosphorylation specifically labels the ($Ca^{2+} + K^+$)-ATPase of sarcoplasmic reticulum [16,23,24], the results obtained from the autoradiograph were consistent with the ATPase activities reported in Table I, suggesting that the sarcolemma and

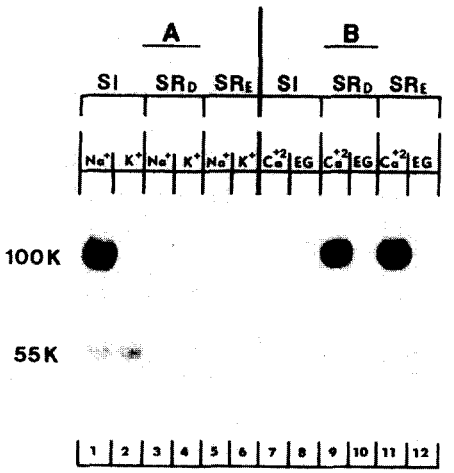

Fig. 1. Autoradiograph of acylphosphoprotein intermediates of ($Na^+ + K^+$)-ATPase and ($Ca^{2+} + K^+$)-ATPase of sarcolemma and sarcoplasmic reticulum fractions. Membranes mentioned in Table I were incubated for 5 s at 5°C with 10 μM [γ-^{32}P]ATP and processed for autoradiography as described previously [16]. For ($Na^+ + K^+$)-ATPase, phosphorylation buffer was 34 mM histidine (pH 7.4), 9 mM $MgCl_2$, 10 mM EGTA, and 100 mM NaCl or 100 mM KCl. For ($Ca^{2+} + K^+$)-ATPase, phosphorylation buffer was 34 mM histidine (pH 7.4), 9 mM $MgCl_2$, 100 mM KCl and 100 μM $CaCl_2$, with or without 10 mM EGTA (EG). Specific activity of [γ-^{32}P]ATP used in panel A was twice that used in panel B. 30 μg of protein were electrophoresed in each lane. Na^+-dependent, trichloroacetic acid-precipitable radioactivity (cpm) applied to panel A lanes was 25 200, 1920 and 0 for sarcolemma, sarcoplasmic reticulum D, and sarcoplasmic reticulum E fractions, respectively. Ca^{2+}-dependent, trichloroacetic acid precipitable radioactivity (cpm) applied to panel B lanes was 681, 18 400 and 21 000 for sarcolemma, sarcoplasmic reticulum D, and sarcoplasmic reticulum E fractions, respectively.

sarcoplasmic reticulum fractions were biochemically distinct. Quantification of the radioactivity incorporated into the acylphosphoprotein intermediates of sarcolemma and sarcoplasmic reticulum by scintillation counting further confirmed the low level of cross-contamination of sarcolemma with sarcoplasmic reticulum (Fig. 1, legend). Visualization of the Coomassie blue stained gel showed a large band of molecular weight 100 000 corresponding to the (Ca^+ + K^+)-ATPase in the two sarcoplasmic reticulum fractions. A protein band of similar molecular weight was much less prominent in the sarcolemma fraction, most probably because the Na^+-pump constitutes a smaller proportion of the total sarcolemma protein (data not shown).

A less intense incorporation of radioactivity into a protein of molecular weight 55 000 was also observed in the sarcolemma fraction, which was not dependent on Na^+ and which was not apparent in the two sarcoplasmic reticulum fractions (Fig. 1, lanes 1 and 2). This protein was identified in our earlier study [16] and probably corresponds to the autophosphorylated regulatory subunit of type II cyclic AMP-dependent protein kinase (R_{II}) (see below). R_{II} is readily autophosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase at low temperature and low ATP concentration [8]. R_{II} was not well visualized in the sarcolemma fraction of panel B, because [γ-^{32}P]ATP of lower specific activity was used to phosphorylate panel B proteins, so that the radioactive intensities of the two ATPase activities in panel A and B would be similar. With a longer exposure time R_{II} was also observed in the sarcolemma fraction of panel B (data not shown).

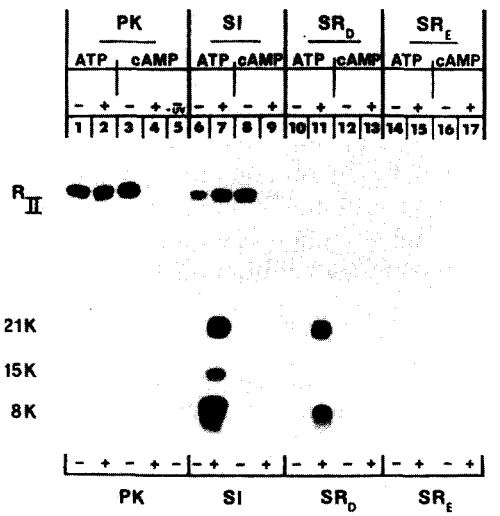

Fig. 2. Autoradiograph showing endogenous phosphorylation by [γ-^{32}P]ATP (ATP), and 8-N_3-[^{32}P]cAMP (cAMP) incorporation into sarcolemma (SI), sarcoplasmic reticulum (SR), and soluble type II cyclic AMP-dependent protein kinase (PK). The same membranes as in Fig. 1 were used. (—) and (+) indicate the absence or presence of non-radioactive cyclic AMP. In lane 5 the soluble cyclic AMP-dependent protein kinase incubated with 8-N_3-[^{32}P]cAMP was not irradiated with ultraviolet light to demonstrate photodependence for probe incorporation. For membrane samples, 15 μg of protein were applied per lane and for soluble protein kinase, 8 μg of protein were applied per lane. A 10% polyacrylamide gel was used.

The fractions mentioned in Table I and Fig. 1 were incubated in the presence of [γ-^{32}P]ATP and cyclic AMP to determine the membrane location of cyclic AMP-dependent protein kinase, and the photoaffinity probe 8-N_3-[^{32}P]cAMP to determine the membrane location of the regulatory subunit of the protein kinase (Fig. 2). Alamethicin was again included to eliminate membrane permeability barriers. Incorporation of 8-N_3-[^{32}P]cAMP into a protein of molecular weight 55 000 was clearly evident in the sarcolemma fraction, and this incorporation was inhibited by an excess of unlabeled cyclic AMP (Fig. 2, lanes 8 and 9). The mobility of the labeled protein was similar to that of the R_{II} subunit of type II cyclic AMP-dependent protein kinase from beef heart [8] (Fig. 2, lane 3). R_{II} subunits were not readily apparent in the sarcoplasmic reticulum D and E fractions (Fig. 2, lanes 12 and 16). The radioactive R_{II} subunits of the membrane fractions were quantified by measuring the difference between covalent incorporation of 8-N_3-[^{32}P]cAMP in the absence and presence of non-radioactive cyclic AMP (Table I). Specific incorporation was found to copurify with that membrane fraction containing the highest activity of (Na^+ + K^+)-ATPase, the sarcolemma.

The intrinsic protein kinase of sarcolemma phosphorylated several proteins in response to added cyclic AMP (Fig. 2, lane 7; proteins 21 000, 15 000 and 8000). In the absence of cyclic AMP only one protein was phosphorylated, the regulatory subunit, R_{II} (Fig. 2, lane 6). Sarcoplasmic reticulum fraction E had no apparent intrinsic protein kinase activity (Fig. 2, lanes 14 and 15), whereas fraction D had an intermediate activity (Fig. 2, lanes 10 and 11), as suggested by the lower level of phosphorylation of proteins of molecular weights 21 000 and 8000. The 15 000-dalton protein was not phosphorylated in fraction D.

Membrane proteins of sarcolemma, and sarcoplasmic reticulum fractions D, and E were phosphorylated in the presence and absence of exogenous soluble type II cyclic AMP-dependent protein kinase to allow an estimate of the efficiency of membrane protein phosphorylation by endogenous protein kinase activity, and to identify the various protein substrates in the three membrane fractions (Fig. 3). A long exposure is shown which reveals most of the phosphorylated proteins. All three membrane fractions had protein substrates of molecular weights 21 000 and 8000, however, phosphorylation of these proteins varied depending upon the addition of soluble protein kinase. Fraction

TABLE II

EFFECT OF SOLUBLE EXTRINSIC PROTEIN KINASE ON MEMBRANE PROTEIN PHOSPHORYLATION OF SARCOLEMMA AND SARCOPLASMIC RETICULUM FRACTIONS

The polyacrylamide gel of Fig. 3 was cut into sections and the radioactivity of the phosphorylated proteins was determined by scintillation counting. Only data for protein bands labeled in the presence of cyclic AMP are shown. Similar results were obtained when the time of phosphorylation was extended to 5 min. Values are pmol $^{32}P_i$ incorporated per mg membrane protein. Abbreviations as per Table I.

Protein phosphorylated	Control			Plus extrinsic protein kinase		
	SI	SR_D	SR_E	SI	SR_D	SR_E
R_{II}	22.2	1.7	0.7	—	—	—
21 000	70.6	31.2	3.3	82.8	310	183
8000	146	45.6	7.1	122	231	141

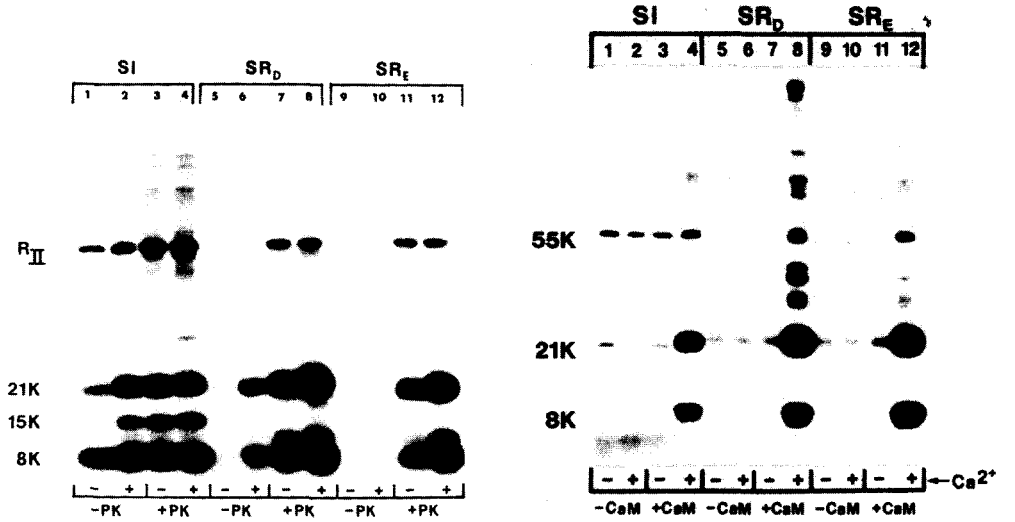

Fig. 3. Autoradiograph showing [γ-^{32}P]ATP phosphorylation of sarcolemma and sarcoplasmic reticulum proteins in the presence and absence of soluble cyclic AMP-dependent protein kinase (PK). 20 μg of membrane protein were phosphorylated in the presence or absence of 60 μg of soluble protein kinase. 10 μg of membrane were then electrophoresed per lane. (—) and (+) indicate the absence or presence of cyclic AMP. A 5—15% linear gradient gel was used. ($Na^+ + K^+$)-ATPase activities of these membranes were 167, 10.2 and 0 μmol P_i mg per h for sarcolemma, sarcoplasmic reticulum D and sarcoplasmic reticulum E fractions, respectively. ($Ca^{2+} + K^+$)-ATPase activities were 9.6, 126 and 199 μmol P_i mg per h for sarcolemma, sarcoplasmic reticulum D and sarcoplasmic reticulum E fractions, respectively.

Fig. 4. Autoradiograph showing [γ-^{32}P]ATP phosphorylation of sarcolemma and sarcoplasmic reticulum proteins in the presence and absence of Ca^{2+} and calmodulin (CaM). The same membrane fractions as in Fig. 3 were used in this experiment; however, they were not pretreated with alamethicin. The radioactive acylphosphoprotein intermediate of the Ca^{2+}-pump (Fig. 1) is not visible in this autoradiograph, because it was hydrolysed in the alkaline buffer used in the Porzio and Pearson [18] electrophoresis system (unpublished observations).

E, for example, had almost no detectable phosphorylation unless exogenous cyclic AMP-dependent protein kinase were added (lanes 9—12), whereas the sarcolemma fraction was almost maximally phosphorylated in the presence of cyclic AMP (lane 2), without the addition of exogenous protein kinase (lane 4). Fraction D exhibited a basal phosphorylation in response to cyclic AMP (lane 6), but this phosphorylation was greatly stimulated by exogenous protein kinase (lane 8). It is also clear that R_{II} was localized predominantly to sarcolemma by comparing its intensity of autophosphorylation in the lanes containing only membrane samples. Autophosphorylated R_{II} visible in the other lanes was contributed by the soluble protein kinase, as demonstrated in Fig. 2. The proteins unique to the sarcolemma fraction having molecular weights other than 21 000 and 8000, which were phosphorylated upon the addition of cyclic AMP, have been identified in our previous studies [11,13].

The intensity of phosphorylation of the proteins of the three membrane fractions in the presence of cyclic AMP was quantitated by scintillation counting (Table II). In the presence of cyclic AMP without soluble protein kinase, the greatest phosphorylation was detected in sarcolemma, although significant phosphorylation did occur in sarcoplasmic reticulum fraction D.

Most notably, addition of soluble protein kinase did not stimulate incorporation of $^{32}P_i$ into sarcolemma proteins appreciably, whereas $^{32}P_i$ incorporation into the sarcoplasmic reticulum proteins was increased 5—50 fold. The amount of phosphorylation of the protein of molecular weight 21 000 (Table II), designated 'phospholamban' by Tada et al. [25], did not seem to correlate well with any of the ATPase activities (Fig. 3, legend). For example, the sarcolemma fraction had 8% or less of the $(Ca^{2+} + K^+)$-ATPase activities of fractions D and E, but it had 30—45% of their phospholamban contents. A similar disparity was apparent for the phosphorylated protein of molecular weight 8000. Endogenous phosphorylation of R_{II}, on the other hand, closely paralleled the $(Na^+ + K^+)$-ATPase activities of the membrane fractions (Table II).

The membrane fractions used to obtain the data shown in Fig. 3 were also examined for endogenous phosphorylation in the presence of Ca^{2+} and calmodulin (Fig. 4). Ca^{2+}- and calmodulin-dependent phosphorylation of the proteins of molecular weights 21 000 and 8000 was detected in all three fractions (lanes 4, 8, and 12), suggesting that both sarcolemma and sarcoplasmic reticulum contain a calmodulin-dependent protein kinase activity. The sarcolemma protein of molecular weight 55 000 phosphorylated in the absence of calmodulin (Fig. 4, lanes 1—3) most probably was R_{II}. As shown in Fig. 2, R_{II} was not present in fractions D or E. However, in the presence of Ca^{2+} plus calmodulin another protein with a molecular weight similar to that of R_{II} was phosphorylated in fractions D and E. The identity of this protein has not yet been established, although preliminary studies have shown that it does not bind cyclic AMP, that it is phosphorylated in the presence of Ca^{2+} plus calmodulin, and that it can be partially purified from Trixton X-100-solubilized membrane vesicles by calmodulin-Sepharose chromatography (Jones, L.R. and Hathaway, D.R., unpublished data). Fraction D also contained several protein substrates for the calmodulin-dependent protein kinase, that were not apparent in the other membrane fractions. The largest of these additional proteins phosphorylated in fraction D (Fig. 4, lane 8) coincided with a Coomassie blue-staining protein band that was observed only in this fraction (data not shown). It was not possible to estimate the molecular weight of this protein accurately, as it migrated with a mobility substantially slower than that of myosin heavy chain, the largest protein standard used.

Discussion

The results of this study demonstrate that cardiac sarcolemma contains an intrinsic type II cyclic AMP-dependent protein kinase activity, which is not present in sarcoplasmic reticulum. The sarcolemma protein kinase activity was identified by its capacity to phosphorylate endogenous protein substrates and also by the properties of its regulatory subunit, detected by autophosphorylation with [γ-^{32}P]ATP and by photoaffinity labeling with 8-N_3-[^{32}P]cAMP. These activities were present in insignificant amounts in the E fraction of sarcoplasmic reticulum. Corbin et al. [26] recently reported that crude membranes from rodent heart contained an intrinsic cyclic AMP-dependent protein kinase activity which phosphorylated exogenously added protein substrates, and they concluded that it was a type II kinase based on its elution from DEAE-cellulose columns.

In a previous study we first presented data suggesting that cardiac sarcolemma contains its own unique protein kinase activity [11]. The increment of membrane phosphorylation stimulated by cyclic AMP has subsequently been shown to be great, ranging between 3- and 9-fold depending on the protein which is being phosphorylated [13]. At least three other groups, however, have suggested that purified cardiac sarcolemma fragments isolated from other species contain low levels of intrinsic protein kinase activity, which is weakly stimulated by added cyclic AMP [27—29]. Much greater membrane phosphorylation was observed in these studies when soluble cyclic AMP-dependent protein kinase was added exogenously [27—29]. Therefore, it was important to document the presence of an intrinsic cyclic AMP-dependent protein kinase activity in sarcolemma membranes in the present study by an independent means, with use of the photoaffinity probe 8-N_3-[^{32}P]cAMP. It is not likely that adsorption of soluble protein kinase activity to the sarcolemma membranes accounted for the activities presently detected, because all membrane fractions were isolated from media containing high concentrations of salts, which should prevent nonspecific binding [26]. Moreover, it seems equally unlikely that a soluble enzyme would be trapped preferentially inside the sarcolemma vesicles.

The sarcoplasmic reticulum D fraction did have a low level of cyclic AMP-dependent protein kinase activity. Several factors could account for this. First, fraction D had more apparent sarcolemma contamination than did fraction E, although the absolute levels of contamination were low. With three separate membrane preparations, fractions D and E were observed to have 7.3 ± 0.9 and 2.3 ± 1.2% (means ± S.E.) of the ($Na^+ + K^+$)-ATPase activities of the corresponding sarcolemma preparations, respectively. R_{II} contents determined with use of 8-N_3-[^{32}P]cAMP, were 6.8 ± 1.4 and 2.6 ± 0.44% of the sarcolemma activities, for the sarcoplasmic reticulum D and E fractions, respectively. Thus the low level of cyclic AMP-dependent protein kinase activity detected in fraction D is consistent with sarcolemma contamination, which may also explain the phosphorylation of protein substrates in fraction D observed to occur in the presence of cyclic AMP alone. The D fraction had the highest content of those protein substrates of molecular weights 21 000 and 8000, which might further facilitate detection of any sarcolemma contamination. However, we cannot exclude the possibility that the D fraction did have a low level of its own protein kinase activity. Preliminary data from our laboratory suggest that the D and E fractions are functionally different by other criteria, for example, by their different responses to the drug ryanodine when active Ca^{2+} transport is monitored [20]. Several investigators have suggested for less pure sarcoplasmic reticulum preparations that these membranes have either minimal [11,30] or substantial [31—33] intrinsic cyclic AMP-dependent protein kinase activity. Different levels of contamination of these preparations with sarcolemma membrane fragments could conceivably account for these discrepancies. In one study the sarcoplasmic reticulum membranes were not extracted with high ionic strength salt solutions, and thus contamination of the sarcoplasmic reticulum fraction with soluble protein kinase may have occurred [32].

Proteins of molecular weights 21 000 and 8000 were phosphorylated by

cyclic AMP-dependent kinase to a significant extent in all three membrane fractions. The relative concentrations of these protein substrates in the sarcolemma and the sarcoplasmic reticulum D and E fractions did not correlate well with either $(Na^+ + K^+)$-ATPase or $(Ca^{2+} + K^+)$-ATPase activities. The protein of molecular weight 21 000 (phospholamban) was originally postulated by Tada et al. [25] to reside in sarcoplasmic reticulum, where it was shown to have a regulatory effect on Ca^{2+}-transport. The present data show that a protein of similar molecular weight may also be localized to sarcolemma, as has been suggested by St. Louis and Sulakhe [29]. However, more sophisticated analysis will be required to establish whether the 21 000 molecular weight protein substrates present in all three membrane fractions are identical. This applies also to the second protein substrate of apparent molecular weight 8000.

The substantial content of a second, lower molecular weight protein substrate present in sarcoplasmic reticulum fractions was suggested in our earlier study [11], and this has recently been confirmed by Bidlack and Shamoo [34]. LePeuch et al. [10] have provided evidence that phospholamban may actually be a dimer of two smaller molecular weight proteins in intact membranes. Most interestingly, phospholamban was also reported to be phosphorylated by an intrinsic Ca^{2+}- and calmodulin protein kinase activity present in the membranes. Our present results are consistent with those of LePeuch et al. [10], suggesting that phospholamban is a substrate for both cyclic AMP-dependent protein kinase and an intrinsic Ca^{2+}- and calmodulin-dependent protein kinase. Our results also suggest that the Ca^{2+}- and calmodulin-dependent protein kinase activity is present both in sarcoplasmic reticulum and in sarcolemma. In addition, the protein kinase activity appears capable of phosphorylating several membrane protein substrates other than phospholamban, as is most apparent in the sarcoplasmic reticulum D fraction. Further studies must be conducted, however, to determine whether a single protein kinase is reponsible for these calmodulin-dependent phosphorylations or whether several calmodulin requiring enzymes are present. Moreover, the subcellular distribution of the calmodulin-dependent activity is less precisely known at present than that of the cyclic AMP-dependent activity. Since the calmodulin experiments were conducted with membranes incubated in the absence of alamethicin, some latent activity may have gone undetected. In other experiments we observed that alamethicin inactivated the calmodulin-dependent activity, as did several non-ionic detergents tested (data not shown). LePeuch et al. [10] have also observed that this enzyme is very labile. Finally, our results suggest that the 21 000-dalton protein substrate of either kinase activity may have other functions besides regulating calcium transport [25], since it is present in high content in an sarcolemma fraction that has low levels of $(Ca^{2+} + K^+)$-ATPase activity.

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