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CHICK HEART PLASMA MEMBRANES

ISOLATION AND ANALYSIS OF AUTOPHOSPHORYLATION

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Plasma membranes have been isolated from hearts of 10-day embryonic and newborn chicks. The membranes obtained were highly enriched in muscarinic acetylcholine receptors, K^+-stimulated, ouabain-sensitive *p*-nitrophenylphosphatase and 5'-nucleotidase. There was little contamination of the membrane fractions by the mitochondrial membranes or by contractile proteins. The autophosphorylation of the isolated membrane fractions was analyzed by measuring ^{32}P incorporation from [γ-^{32}P]ATP into total membrane protein and into individual membrane components. Membranes obtained from embryonic hearts contained significantly more cAMP-dependent and -independent protein kinase activities than membranes from newborn chick hearts. Treatment of the membranes with Triton X-100 or the peptide ionophore alamethicin increased phosphorylation in membranes from either newborn or embryonic hearts. Membranes from embryonic hearts contained substrates for membrane-bound cAMP-dependent and -independent protein kinases either not observed or present in low amount in membranes from newborn hearts, and vice-versa. Notably, a 38 kDa protein was markedly phosphorylated by endogenous cAMP dependent protein kinase in plasma membrane enriched fractions from embryonic hearts. This phosphoprotein was not easily detected in any fraction obtained from newborn hearts. One cAMP-dependent phosphoprotein had an M_r of 27000 or 11000, depending on the conditions used to solubilize it. This protein was present in sarcolemma-enriched membranes as well as membrane fractions containing sarcoplasmic reticulum. There was more of this phosphoprotein in newborn heart membranes than in embryonic hearts. The phosphorylation of this protein was markedly enhanced by the peptide ionophore alamethicin. A second cAMP-dependent phosphoprotein with an M_r of 27000 was also detected in the sarcolemma-enriched membranes.

Introduction

The molecular basis of the positive inotropic actions of catecholamines and calcium on cardiac tissue have not yet been satisfactorily elucidated. It is clear calcium must be supplied to and removed from the contractile elements in order to insure proper cardiac function. There are multiple mechanisms regulating calcium movements in cardiac tissue. This is in contrast to skeletal muscle in which the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase appears to be the main activity regulating the Ca^{2+} concentration inside the cell. In cardiac tissue, in addition to a sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase, there are several activities at the level of the sarcolemma

Abbreviations: EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Mops, morpholinopropanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

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that seem to be important in regulating Ca^{2+} movements. These include a sarcolemma (Ca^{2+} + Mg^{2+})-ATPase [1] which has properties distinct from the sarcoplasmic reticulum enzyme [2,3], an Na^{+}-Ca^{2+} exchange mechanism [4,5], a voltage-dependent Ca^{2+} channel [6] and Ca^{2+}-binding activity which seems to involve, at least in part, phospholipids [7].

Protein phosphorylation may play a role in the regulation of cardiac contractile force. Catecholamines elevate cAMP whose only well documented effect in higher eukaryotic cells is to activate a cAMP-dependent protein kinase (for reviews, see Refs. 8 and 9). Furthermore, Ca^{2+} is known to activate a variety of other cardiac protein kinases [10–13]. If protein phosphorylation is involved in the response of the heart to inotropic agents, protein components of one or more of the Ca^{2+}-regulating mechanisms would seem to be likely substrates. The cAMP-dependent and Ca^{2+}-dependent phosphorylation of a sarcoplasmic reticulum protein with an M_r of 22000 is well documented [11,14–16]. This protein is termed phospholamban and is believed to modulate the activity of the cardiac sarcoplasmic reticulum Ca^{2+}-ATPase. It now appears that a protein similar in size to phospholamban may be a component of cardiac sarcolemma and possibly be involved in regulating sarcolemma Ca^{2+} fluxes [17–19]. However, there is some controversy concerning the identity of the protein(s) being studied [20]. In any event, Walsh et al. [21] have shown that epinephrine induces the phosphorylation of a similar sized (M_r 27000) sarcolemma protein in the perfused rat heart. However, these authors found the phosphorylation of the 27 kDa protein to lag behind the inotropic response to epinephrine. Another site regulated by phosphorylation appears to involve the sarcolemma Ca^{2+}-ATPase since a recent report showed that the activity of the sarcolemma Ca^{2+}-ATPase could be regulated in vitro under phosphorylating conditions [22]. Direct phosphorylation of the enzyme or a modifier protein was not demonstrated.

The developing chick heart provides an interesting model to study the role of membrane protein phosphorylation in the inotropic responses of the heart. We have previously demonstrated that cAMP levels are several-fold higher in 7–9-day embryonic hearts compared to newborn (0–2 wk) hearts [23]. The elevated cAMP in embryonic hearts results in the activation of the soluble cAMP-dependent protein kinase, such that 60% appears active in 7-day embryonic hearts compared to approx. 25% in newborn hearts [23]. At the same developmental time that cAMP levels are elevated and cAMP-dependent protein kinase activated, the hearts exhibit an increased sensitivity to the positive inotropic effect of calcium. Newborn hearts have an EC_{50} value (concentration producing 50% maximal response) for calcium of 3 mM, while 7–9-day embryonic hearts and 4-day embryonic hearts have EC_{50} values for calcium of 0.6 and 0.3 mM, respectively [24]. Others have shown that the developing chick heart also exhibits differential responses to catecholamines and acetylcholine [25–28]. Thus, the chick heart can be studied at different development stages when it contains varying levels of cAMP and exhibits differential responses to various inotropic agents. For these reasons it should be an excellent system to study the role of membrane protein phosphorylation in cardiac function. In order to do so, the present initial study was undertaken to isolate and characterize membranes from embryonic and newborn chick hearts and to analyze the autophosphorylation reactions which occur in these membranes.

Experimental procedures

Materials. Fertilized eggs were purchased from SPAFAS, Inc., Roanoke, IL. Ultra-pure sucrose was obtained from Schwarz/Mann. [γ-^{32}P]ATP, [^{3}H]quinuclidinyl benzilate, ^{3}H-labeled 5′-adenosine monophosphate and ACS (aqueous counting scintillant) were purchased from Amersham/Searle. Atropine sulfate, Trizma base, Mops, Hepes, adenosine triphosphate, cytochrome *c*, *p*-nitrophenyl phosphate, ovalbumin, phosphorylase *a*, myoglobin, bovine serum albumin, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, and acrylamide were obtained from Sigma. Cyclic AMP was from P-L Biochemicals. Reagents for SDS-polyacrylamide gel electrophoresis were purchased from BioRad. Triton X-100 was from Research Products International. Alamethicin was kindly provided by Dr. G.B. Whitfield of the Upjohn Company. All other reagents were from

commercial sources and were reagent grade or better.

Membrane isolation. Hearts were removed from newborn chicks (0–10-days old) or 10-day old embryos and processed as previously described [32]. Sucrose gradients were made according to method 2 [32], using a 5-step discontinuous gradient (15/28/32/36/40% sucrose). Centrifugation of the gradients was reduced from 16 to 3 h in later experiments with no change in the results. Five fractions were obtained at the interfaces and are designated (interface in parentheses) T1 (15/28), T2 (28/32), T3 (32/36), T4 (36/40), and T5 (pellet). The fractions were either stored briefly at −20°C or, if for longer periods, in liquid N_2.

Membrane marker assays. The muscarinic acetylcholine receptor, a putative sarcolemma marker, was assayed by analyzing the fractions for the binding of the antagonist ligand [^{3}H]quinuclidinyl benzilate, as previously described [32].

K^+-stimulated, ouabain-sensitive *p*-nitrophenylphosphatase activity, an expression of the ($Na^+ + K^+$)-ATPase, another sarcolemma marker, was assayed in a reaction containing 50 mM Tris-HCl, pH 7.8, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM *p*-nitrophenyl phosphate, ±20 mM KCl, ±1 mM ouabain and 20–100 μg protein in a final volume of 1 ml. The reaction was preincubated minus the *p*-nitrophenyl phosphate substrate for 5 min at 37°C, the substrate was added and the reaction incubated 30 min longer. Reactions were stopped with 0.1 ml 50% trichloroacetic acid; 2 ml 0.5 M Tris base were added and the A_{410} was determined [33]. The reaction was linear with time and protein concentration. All K^+-stimulated activity was completely inhibited by ouabain.

5′-Nucleotidase activity was determined as described [34] in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM ^{3}H-labeled 5′-AMP (40000 cpm) and membrane protein (1–3 μg) or homogenate or pellet (5–20 μg protein) at 30°C for 30 min in a total volumne of 0.2 ml. [^{3}H]Adenosine was isolated on small DEAE-Sephadex A-25 columns [34]. Activity was linear with time and protein concentration and totally inhibitable by α,β-methylene ADP [35].

Succinate dehydrogenase was assayed spectrophotometrically by following the reduction of cytochrome *c* [36] except that 1 mM EDTA was substituted for bovine serum albumin.

Ca^{2+}-loading experiments. Fractions T1, T2, T3, and T4 from newborn hearts were incubated with a Ca^{2+}/oxalate medium in order to separate sarcoplasmic reticulum from sarcolemma in a method modified slightly from Jones et al. [29]. The incubation mixture contained (in a final volume of 0.5 ml): 50 mM Hepes, pH 7.2, 30 mM $MgCl_2$, 15 mM EGTA, 100 mM KCl, 15 mM $CaCl_2$, 20 mM ATP, 2.5 mM oxalate (K^+), 30 mM creatine phosphate, 0.25 mg/ml creatine phosphokinase and 1 mg membrane protein. The reaction was incubated at 37°C for 5 min, an additional aliquot of oxalate (to bring it to 5 mM) was added and the reaction was incubated for 5 min longer, and then placed on ice. The Ca^{2+}-loaded membranes were layered onto sucrose gradients consisting of 1 ml 28% sucrose, 3 ml of the sucrose solution on top of which they were originally harvested and 1 ml 40% sucrose. The gradients were centrifuged for 2 h in an SW50.1 rotor at 45000 rpm. The fractions were collected at the interfaces.

SDS-gel electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed on slabs according to Laemmli [37], except that the sample buffer was modified such that it contained (final concn.) 67 mM Tris-phosphate, pH 6.8, 2% SDS, 10% glycerol, 3.3% β-mercaptoethanol, 3.3 mM EDTA and 0.002% Bromophenol blue. The samples were dissolved in this buffer and treated as described in the figure legends. Electrophoresis was performed at 30 mA/slab until the dye front was 0.5 cm from the bottom. Molecular weight markers used were β-galactosidase (135000), phosphorylase *a* (94000), lactoperoxidase (77000), bovine serum albumin (67000), ovalbumin (43000), glyceraldehyde-3-phosphate dehydrogenase (36500), carbonic anhydrase (29000), myoglobin (17600) and cytochrome *c* (12500). The gels were fixed in 40% isopropanol/10% acetic acid for 1 h with shaking, stained in 25% isopropanol/10% acetic acid/0.1% Coomassie blue R for 45 min, and destained in 10% isopropanol/10% acetic acid overnight. The gels were dried under heat and vacuum. Where applicable, radioautograms were prepared of the dried gels using Kodak No-Screen X-ray film.

Protein phosphorylation assays. Protein phosphorylation was determined by analyzing the transfer of ^{32}P from [γ-^{32}P]ATP to protein in a reaction that contained: 50 mM morpholinopropanesulfonic acid (Mops), pH 7.2, 7.5 mM magnesium acetate, 2 mM EGTA, 0.3 mM [γ-^{32}P]ATP (100–200 cpm/pmol), ± 10 μM cAMP, and membrane protein in a final volume of 0.04 ml. Where indicated, 0.1% Triton X-100 was added to the assays. In other experiments, membranes were preincubated for 20 min at room temperature with equal concentrations (w/w) of alamethicin dissolved in ethanol (38) or ethanol only. The concentration of ethanol during the preincubation was 1%. The reactions were incubated at 30°C for the times specified. ^{32}P incorporation into total membrane protein was quantitated by an acid precipitation assay performed as described by LePeuch et al. [11], except that the final protein pellet was dissolved in 0.1 ml of formic acid (88%) and radioactivity determined by scintillation counting using 3.5 ml ACS/sample.

^{32}P incorporation into individual membrane proteins was determined using SDS-polyacrylamide gel electrophoresis in combination with radioautography. In these experiments, the phosphorylation reaction was stopped by the addition of the SDS-sample buffer and heating as described in the figure legends.

Results

Membrane isolation

Initial experiments designed to isolate membranes from newborn and embryonic chick hearts were performed essentially according to the method described for these hearts by Kutchai et al. [39]. This method utilized low salt buffers and a top-loaded 2-step discontinuous sucrose gradient (25% and 40%). However, we did not obtain satisfactory results using this method whether or not 0.3 M KCl was added to the sucrose gradient solutions [40]. Several other published procedures for isolating cardiac membranes from hearts of other species [29,30,41,42] were also tested but gave unsatisfactory results.

We therefore devised a procedure utilizing a low salt/sucrose extract and an expanded discontinuous sucrose gradient to separate sarcolemma from other membranes and contractile proteins [32]. Briefly, a high speed pellet is obtained from a homogenate made in 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol (buffer A) containing 0.25 M sucrose. The resuspended pellet is applied to the top of a 5-step discontinuous gradient and processed as described in Methods. Five fractions labelled T1, T2, T3, T4, and T5 (lighest to heaviest) were obtained [32].

TABLE I

K^+-STIMULATED, OUABAIN-SENSITIVE *p*-NITROPHENYLPHOSPHATASE ACTIVITIES IN FRACTIONS OBTAINED FROM NEWBORN AND EMBRYONIC CHICK HEARTS

'Untreated' refers to samples used in the assay as described in Methods. Samples under '+SDS' were pretreated with 0.025% SDS at room temperature for 20 min prior to the assay. The final concentration of SDS in the actual *p*-nitrophenylphosphatase reaction was 0.001%. *n* is number of determinations. n.d., not determined.

Fraction	μmol *p*-nitrophenol produced/mg protein per h			
	Newborn		Embryo	
	Untreated ($n=6$)	+SDS ($n=3$)	Untreated ($n=4$)	+SDS ($n=2$)
T1	2.77±0.32	5.18±0.64	0.96±0.23	2.11±0.40
T2	2.57±0.42	3.17±0.80	1.52±0.33	2.40±0.79
T3	1.42±0.36	2.16±0.12	1.33±0.23	1.67±0.14
T4	0.55±0.26	0.53±0.07	0.63±0.18	0.30±0.15
T5	0.38±0.12	0.18±0.05	0.50±0.12	n.d.
Homogenate	0.31±0.06	0.37±0.03	0.27±0.11	0.33±0.03

Characterization of isolated membranes

Embryonic and newborn chick heart sarcolemma were successfully separated from the heavier mitochondrial membranes and contractile proteins using the five step discontinuous sucrose gradients. The fractionation of $(Na^+ + K^+)$-ATPase, measured as *p*-nitrophenylphosphatase, is summarized in Table I. Activity was measured in membranes with and without pretreatment with 0.25% SDS to unmask latent activity [43]. After pretreatment with SDS, the highest specific activity was found in T1 and T2 in both newborn and embryonic hearts to unmask latent activity [43].

The enrichment in activity in T1 and T2 was 19- and 12-fold in SDS-treated fractions from newborn hearts and 6- and 7-fold in similarly treated fractions from embryonic hearts. The recovery of activity in T1 and T2 totalled 15–20% in newborn hearts and 9–24% in embryonic hearts. A large fraction of the activity (25–50%) was found with low specific activity in T5, most likely nonspecifically absorbed to the 'sticky' contractile proteins.

Another sarcolemma marker, 5'-nucleotidase, was found to be enriched in T1 in fractions obtained from either newborn and embryonic hearts (Table II). The activity was enriched 34-fold and 23-fold in T1 from newborn and embryonic hearts, respectively. This activity represents in each case approx. 7% of homogenate activity.

The distribution of the muscarinic acetylcholine receptor in the five fractions obtained from embryonic and newborn hearts has been recently reported by us [32]. The receptor is enriched 14- and 12-fold in T1 and T2 from newborn hearts and 5-fold in T1 and T2 in embryonic hearts. Recoveries were 3–7% in T1 and T2 [32].

TABLE II

5'-NUCLEOTIDASE ACTIVITY IN FRACTIONS OBTAINED FROM NEWBORN AND EMBRYONIC HEARTS

Fraction	nmol AMP hydrolyzed/min per mg protein	
	Newborn ($n=6$)	Embryo ($n=4$)
T1	140.3 ± 17.7	92.7 ± 12.1
T2	66.0 ± 12.6	29.7 ± 5.3
T3	45.3 ± 3.6	20.9 ± 3.3
T4	18.7 ± 4.4	14.5 ± 2.8
T5	2.7 ± 0.8	5.4 ± 1.6
Homogenate	4.9 ± 1.3	4.1 ± 0.8

TABLE III

SUCCINATE DEHYDROGENASE ACTIVITY IN PARTICULATE FRACTIONS FROM NEWBORN AND EMBRYONIC CHICK HEARTS

The number in parentheses gives the number of determinations.

Fraction	nmol cytochrome *c* reduced/mg protein per min	
	Newborn	Embryo
T1	18.4 ± 4.0 (9)	12.6 ± 4.1 (6)
T2	22.1 ± 5.4 (7)	16.4 ± 3.8 (7)
T3	56.6 ± 9.5 (8)	33.2 ± 9.0 (7)
T4	278.5 ± 44.8 (9)	76.4 ± 19.2 (8)
T5	177.1 ± 26.6 (7)	97.8 ± 20.0 (8)
Homogenate	144.9 ± 9.5 (8)	62.3 ± 12.4 (8)

Sialic acid content was measured [44] in a few experiments and found concentrated in T1 and T2 in newborn hearts (60–80 nmol/mg protein) with enrichment ranging from 20- to 50-fold.

The specific activity of succinate dehydrogenase (Table III) in T1 and T2 from the newborns was less than 0.15 of the homogenate and the total amount in these fractions accounted for less than 0.04 and 0.11% of that in the material applied to the gradients. Similarly, the specific activity of succinate dehydrogenase in T1 and T2 from embryonic hearts was less than 0.2–0.25 that of the homogenate and accounted for less than 0.1–0.26% of the total activity in the material applied to the gradient.

Sarcoplasmic reticulum is difficult to accurately detect since there is no good 'marker' enzyme or protein unique to this membrane. $(Ca^{2+} + Mg^{2+})$-ATPase is not a good marker because $(Ca^{2+} + Mg^{2+})$-ATPase activity is present in both sarcoplasmic reticulum and sarcolemma [1–3] membranes. Several investigators [1,2,19,22,29] have used a technique developed by Jones et al. [29] to load sarcoplasmic reticulum vesicles with Ca^{2+} in the presence of oxalate so that they become heavier than sarcolemma vesicles on sucrose gradients. Using this technique, only T2 migrated

as a heavier species on sucrose gradients. This suggests that T2 contains sarcolemma contaminated with sarcoplasmic reticulum membranes.

Autophosphorylation of membrane fractions

The autophosphorylation of total membrane protein in the isolated membrane fractions was determined using [γ-^{32}P]ATP to measure ^{32}P incorporation into acid-precipitable proteins [11]. Phosphorylation was found to be linear with protein concentration only with very low concentrations of protein. At protein concentrations greater than 0.15 mg/ml phosphorylation appeared to decrease with increasing protein concentration (data not shown). Therefore, routine assays contained between 3 and 5 μg protein (0.07–0.125 mg protein/ml). Phosphorylation was rapid and was maximal after 2–5 min at 30°C.

In sarcolemma-containing fractions T1 and T2 from embryonic hearts autophosphorylation was readily detectable. Each fraction contained cAMP-dependent and -independent activity. However, initial attempts to demonstrate autophosphorylation of membrane proteins in fractions T1 and T2 from newborn heart gave results of extremely low levels of autophosphorylation. We reasoned that this might be caused at least in part by the membranes being present in sealed vesicles. This is suggested by our results on *p*-nitrophenylphosphatase activities (Table I). Sonication of the membranes for 1 min prior to use for autophosphorylation did not lead to enhanced phosphorylation. However, the addition of Triton X-100, or the peptide ionophore alamethicin, did enhance autophosphorylation of membrane proteins. The results of experiments comparing autophosphorylation of the T1 and T2 fractions obtained from newborn and embryonic hearts are shown in Table IV. In these experiments autophosphorylation was measured in the presence and absence of cAMP under control additions (no additions or pretreatments), with 0.1% Triton X-100, and with membranes pretreated for 20 min at room temperature with alamethicin (1:1 on a weight basis) [38]. Since the alamethicin was dissolved in ethanol, experiments which contained similar amounts of ethanol only were performed and gave results no different than control conditions (data not shown).

Several points in Table IV should be noted. Autophosphorylation of membrane proteins was present in all membranes fractions. Membrane phosphorylation was enhanced by Triton X-100 and by alamethicin in all cases tested. The degree of enhancement was similar with either agent, except alamethicin appeared to be much more potent than Triton in the T1-newborn fraction. In

TABLE IV

AUTOPHOSPHORYLATION OF MEMBRANE PROTEINS IN FRACTIONS OBTAINED FROM EMBRYONIC AND NEWBORN CHICK HEARTS

All reactions contained 5 μg protein and were incubated 2 min at 30°C. Other conditions are described in Methods. n.d., not determined.

Fraction	pmol ^{32}P incorporated/mg protein per 2 min					
	Control		+Triton X-100		+Alamethicin	
	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP
T1						
Embryo	1941	2120	3606	4558	3863	4580
Newborn	673	908	970	1481	4688	5635
T2						
Embryo	747	1037	1091	1402	n.d.	n.d.
Newborn	403	471	687	696	1825	n.d.

most instances membranes from embryonic hearts were autophosphorylated to a greater degree than those from newborn hearts. cAMP-dependent protein kinase and appropriate substrates appear to be present in most of the fractions. However, where it appears absent, it is possible that cAMP-dependent activities could be masked by other protein kinase activities.

In additional experiments, histone (Sigma type IIAS, 1.2 mg/ml) and casein (2.0 mg/ml) were tested as exogenous substrates of the membrane bound protein kinases. In these experiments, the amount of autophosphorylation of the membranes alone were substracted from that obtained in the presencce of histone. Appreciable amounts of cAMP-dependent histone kinase activity were found in T1 from embryonic hearts. The histone kinase activity in fraction T1-embryo was 2623 and 3725 pmol ^{32}P incorporated/mg protein per 2 min in the presence and absence of cAMP, respectively. The T2 fraction from embryonic and T1 and T2 from newborn hearts had much less activity toward histone. Casein was not appreciably phosphorylated by any of the fractions under the conditions utilized.

Analysis of autophosphorylation of membrane proteins by SDS-polyacrylamide gel electrophoresis and radioautography

The polypeptide profile of the isolated mem-

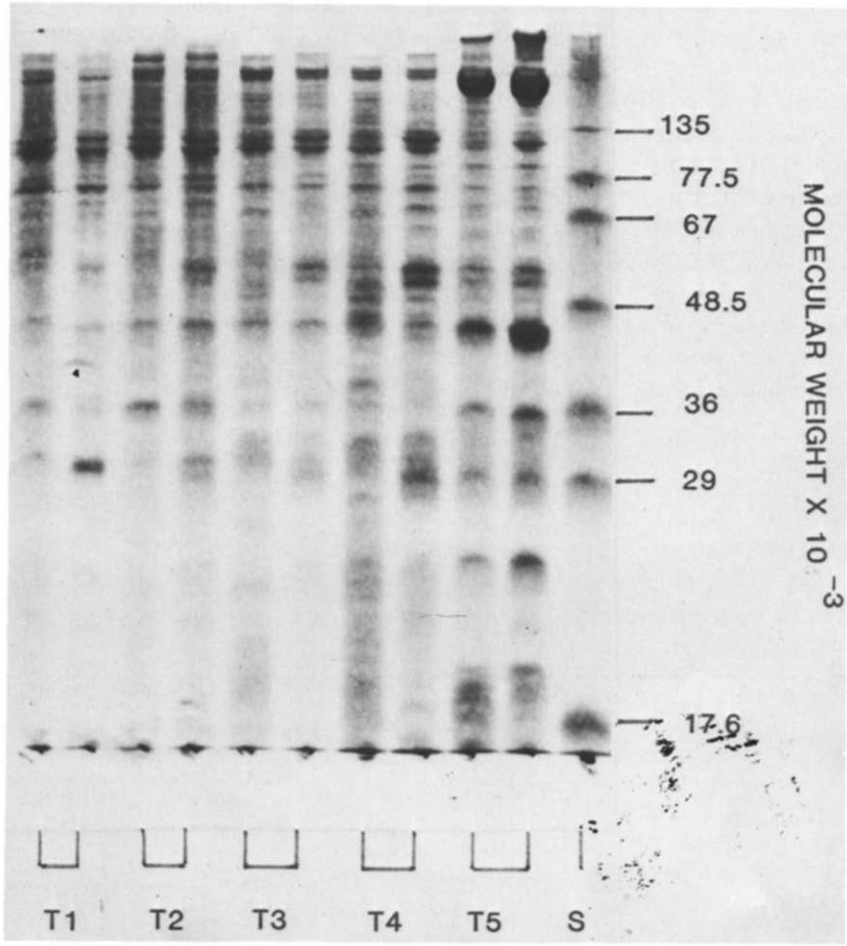

Fig. 1. SDS-polyacrylamide gel of membrane fractions isolated from embryonic and newborn chick hearts. The gel contained 10% acrylamide. T1, T2, etc. refers to the fractions obtained from the sucrose gradients as described in Methods. In each pair, the embryonic fraction is on the left and the newborn fraction on the right. S denotes the molecular weight markers.

brane fractions as seen in an SDS gel containing 10% polyacrylamide is shown in Fig. 1. It is clear that the fractions T1 and T2 are essentially free of myosin and actin, which are the prominent peptides seen in the T5 fraction. There are certain similarities as well as differences between the analogous fractions obtained from the embryonic and newborn hearts. For example the polypeptide of approx. 31 kDa that is prominent in T1-newborn is more or less absent in the analogous embryonic fraction.

The autophosphorylation of membrane proteins in T1 and T2 was performed in the presence and absence of cAMP and analyzed in radioautograms prepared from 14% gels (Figs. 2 and 3). The 14% gels were used because of the interest in phosphoproteins of lower M_r [11,17–19]. Two sets of experiments are shown. The experiments in Fig. 2 were 'control' experiments, while those in Fig. 3 contained 0.1% Triton X-100. The results show that membrane phosphorylation is greater in the fractions from embryonic hearts than in those from newborn hearts. This is true for untreated or control membranes (Fig. 2) and for reactions containing 0.1% Triton X-100 (Fig. 3). This confirms the data obtained from the acid precipitation analyses of total membrane protein phosphorylation (Table IV).

Several notable differences are evident in the cAMP-dependent as well as -independent phosphorylations. In the untreated T1-embryonic membranes, peptides of M_r 38000 and 16000 are phosphorylated by an endogenous cAMP-dependent protein kinase (Fig. 2, A). In contrast, the T1-newborn membranes appear to lack the 38000 substrate but contain proteins of 27000 and 11000 whose phosphorylation is cAMP-dependent (Fig. 2, B). In the presence of Triton X-100, proteins in T1-embryonic membranes with M_r of 34000 and, to a lesser degree, 25500 and 11000, become good substrates of the cAMP-dependent protein kinase (Fig. 3, A). In T1-newborn membranes, Triton enhanced the overall phosphorylation of cAMP-

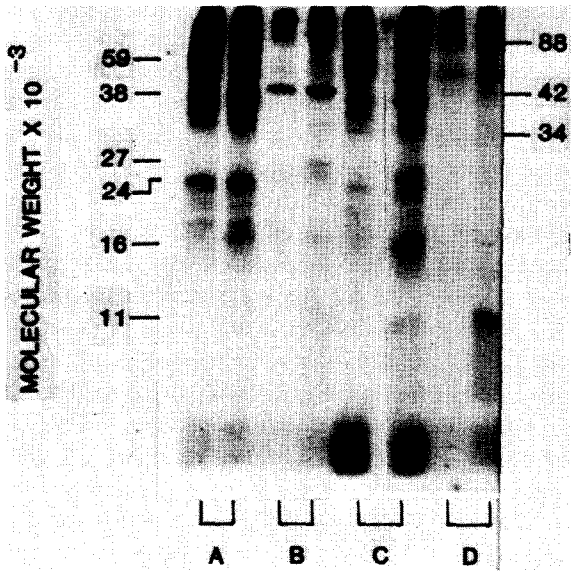

Fig. 2. Radioautogram depicting the autophosphorylation of membrane proteins in fractions obtained from newborn and embryonic hearts. Membranes were phosphorylated for 5 min at 30°C under the conditions listed in Methods. Phosphorylation was terminated by adding the SDS-sample buffer and boiling the samples for 1 min. Each pair of samples represents reactions run in the absence (left sample) and presence (right sample) of cAMP. Each reaction contained 10 g protein. The specific activity of [γ-^{32}P]ATP was 333 cpm/pmol. The radioautogram was prepared from a 14% gel and was exposed 5 days. A, T1-embryo; B, T1-newborn; C, T2-embryo; D, T2-newborn.

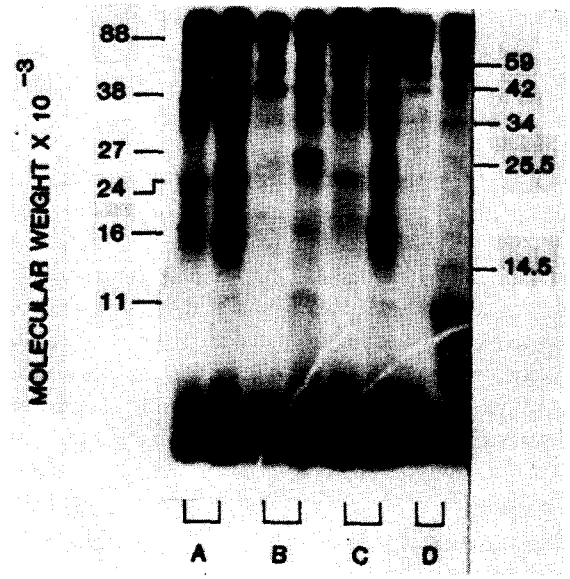

Fig. 3. Radioautogram depicting the autophosphorylation of membrane proteins in fractions from newborn and embryonic hearts. The conditions and fractions are as listed under Fig. 2, except that 0.1% Triton X-100 was present during all phosphorylation reactions.

dependent protein kinase substrates (59, 34, 27, 25.5, 16 and 11 kDa) (Fig. 3, B). Higher molecular weight substrates are not well resolved in these 14% gels. However, in 5–20% gradient gels, a protein of $M_r = 160000$ in T1 membranes was found to be phosphorylated by the endogenous cAMP dependent protein kinase (data not shown).

Differences in the cAMP-independent reactions are also evident in the T1 fractions. The newborn T1 fractions contains a prominent phosphopeptide of approx. $M_r = 43000$ (Fig. 2, B) not apparent in embryonic fractions. That this phosphopeptide is not actin is easily demonstrated on lower percentage gels (data not shown). Triton X-100 treatment led to enhanced phosphorylation of cAMP-independent reactions in both embryonic and newborn T1 fractions (Fig. 3, A and B).

The T2-embryonic fraction appears qualitatively similar in its phosphopeptide profile to the T1 fraction. It appears to contain more of the 25.5, 16 and 11 kDa substrates of the cAMP-dependent protein kinase in the control membranes (compare Fig. 2, C and A) and less of the 24 kDa cAMP-independent kinase substrate. The major cAMP-dependent protein kinase substrate in T2-newborn has an M_r of 11000 (Fig. 2, D and Fig. 3, D). The amount of 11 kDa phosphoprotein in newborn fractions exceeds that in embryonic fractions (Fig. 2 and 3, compare D and C). In the presence of Triton, overall phosphorylation of the T2 fractions is enhanced (Fig. 3, C and D). In addition, a 14.5 kDa substrate appears in the T2-newborn which is not present in T2-embryo or T1-newborn or embryo (Fig. 3, compare D with A, B, and C).

The M_r of the cAMP-dependent phosphopeptide at 11000 seen in Figs. 2 and 3 depended on the conditions used to prepare the sample for electrophoresis [17,18]. We analyzed these differences in fractions T1 and T2 and found an interesting result. We found that there were two distinct proteins with similar molecular weights, which were both autophosphorylated in the presence of cAMP in vitro. One protein, more apparent in T2, had an M_r of approx. 27000 if the sample was incubated in SDS sample buffer overnight at room temperature. But if the sample as boiled 1 min at 100°C, as was done for the samples in Figs. 2 and 3, the M_r changed to 11000. Another phosphoprotein was present in T1 and had an M_r only slightly smaller than the 27000 protein in T2, but its M_r did not change upon heating. This protein is apparent in Lane B (plus cAMP) in Fig. 3. Recently, we have found that both proteins are phosphorylated in the intact heart in response to the β-adrenergic agonist isoproterenol (Hosey, M.M., unpublished data).

Since Triton X-100 and alamethicin increased overall protein autophosphorylation (Table IV) we compared the effects of these agents on the autophosphorylation of individual membrane proteins. Qualitatively, the effects of Triton and alamethicin were similar, but quantitatively there were apparent differences. Alamethicin was much more effective in stimulating the phosphorylation of the 11 and 14.5 kDa peptides than Triton. No differences in the ability of the two agents to affect cAMP-independent reactions were observed.

Discussion

The purpose of this investigation was to develop a reliable procedure to isolate plasma membranes from newborn and embryonic chick hearts and to analyze the autophosphorylation of the isolated membranes. The high salt extraction conditions used in many cardiac membrane isolation procedures to solubilize contractile proteins might lead to extraction of membrane proteins. This is well documented for the erythrocyte membranes. We felt it imperative to avoid using high salt in our membrane isolation since it conceivably could have had differential effects on embryonic compared to newborn membrane proteins. We have obtained cardiac membrane proteins enriched many-fold in muscarinic acetylcholine receptors, $(Na^+ + K^+)$-ATPase and 5′-nucleotidase, from low salt extracts of newborn and 10-day embryonic chick hearts.

The T1 and T2 contained the highest specific activities of sarcolemma proteins, but T2 appears to also contain the sarcoplasmic reticulum membranes. Sarcolemma marker proteins with similar specific activities were found in fraction T3 from embryonic hearts but the origin of T3 membranes is not clear. The specific activities of [^{3}H]quinuclidinyl benzilate binding and of ouabain sensitive *p*-nitrophenylphosphatase that we obtain in fractions T1 and T2 in embryonic hearts are very

similar to those obtained for sarcolemma fractions from 14-day embryonic hearts by other investigators [46,47].

The interesting finding is that membrane fractions from embryonic heart contains significantly more protein kinase activities and substrates for these enzymes than analogous fractions from newborn hearts. In addition, these preparations appear to contain more endogenous membrane-bound protein kinase activities than cardiac membrane preparations obtained by others [17–19,29].

Most investigations of cardiac membrane protein phosphorylation have had to rely on the use of exogenous protein kinases to catalyze the reactions [11,18,19,29]. Our preparations of T1 and T2 from embryonic hearts appear to be the most active autophosphorylating cardiac membranes reported to date. This observation plus our previous results showing that embryonic hearts contain elevated levels of cAMP and consequently, activated cAMP-dependent protein kinase [23], suggest that the membranes of embryonic hearts may be highly phosphorylated in vivo.

The membrane fractions from these actively autophosphorylating embryonic T1 and T2 fractions contain a prominent cAMP-dependent phosphoprotein of 38 kDa which is not easily detected in any fraction from newborn hearts nor apparently present in cardiac membrane fractions of other species [17–19]. The identity of this protein is not apparent at this time.

Our results confirm that there is a heat-dependent interconversion of a membrane protein from 27 kDa to 11 kDa [17,18]. This protein is a good substrate for the endogenous cAMP-dependent protein kinase. This 27/11 kDa phosphoprotein is more evident in membranes from newborn chick hearts that embryonic hearts. It is present in the most enriched sarcolemma fraction (T1) as well as that fraction also containing sarcoplasmic reticulum (T2). However, another protein phosphorylated in the presence of cAMP also has an M_r close to 27000 and is seen in T1. Its M_r does not change on heating. The cAMP-dependent autophosphorylation of the 11 kDa protein in T1 and T2 is markedly potentiated by alamethicin. In addition to being a substrate for cAMP-dependent protein kinase, the 11 kDa protein is also phosphorylated by a membrane-bound Ca^{2+}/calmodulin-dependent protein kinase (Hosey, M.M., unpublished data).

Earlier evidence has suggested that there are cAMP-dependent phosphoprotein(s) in cardiac sarcolemma that modulate Ca^{2+} transport at this membrane [50–52]. Lamers and co-workers [17,18] showed that highly purified sarcolemma contained a heat-dependent interconvertible 24/9.5 kDa cAMP-dependent phosphoprotein. This protein appears to modulate the sarcolemma (Ca^{2+} $+Mg^{2+}$)-ATPase to a small extent. In a brief report, Demaille and co-workers [19] showed that a 23 kDa sarcolemma protein could be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase and might be involved in modulating the Ca^{2+} channel. They suggested, but did not demonstrate, that this 23 kDa protein was the same as what they previously termed sarcoplasmic reticulum phospholamban and as Lamers 24/9.5 kDa protein [11,49]. However, in their hands, what they have called phospholamban [11], but not the 23 kDa sarcolemma protein [19], was also a substrate for a Ca^{2+}/calmodulin-dependent protein kinase. The issue is further confused by the findings of Collins et al. [20] who showed that the amino acid composition and electrophoretic behavior of their phospholamban [20] bore no resemblance to that of Demaille and co-workers [11,49]. Our data suggests the presence of two cAMP-dependent phosphoproteins with similar molecular weights, only one of which is converted to a smaller species by heating. Whether or not the 27/11 kDa protein that we and others are studying is the same or different in sarcolemma and sarcoplasmic reticulum is not yet clear. More important, the function of the phosphoprotein(s) in regulating Ca^{2+} movements at the level of the sarcolemma needs to be rigorously studied. Studies of catecholamine-stimulated membrane phosphorylation in intact embryonic and newborn chick hearts are underway in our laboratory and should help to discern which cardiac membrane proteins are phosphorylated under physiological conditions.

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