Biochimica et Biophysica Acta, 553 (1979) 197—212

BBA 78366

MASS ISOLATION OF CELL SURFACE MEMBRANE FRAGMENTS FROM PIGEON HEART

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(Received September 6th, 1978)

Key words. ATPase, Membrane fragment isolation, Cell surface, Concanavalin A binding, (Pigeon heart)

Summary

Cell surface membrane fragments were isolated and purified by successive rate zonal and isopycnic centrifugation of calcium oxalate-loaded pigeon heart microsomes in sucrose density gradients. The most highly purified cell membrane fraction sediments at a buoyant density of 1.105 g/ml. Some of the membrane pieces are present as open fragments and leaky vesicles, while others form tightly sealed vesicles of both inside-in and inside-out membrane orientation. The pigeon heart cell membrane preparation exhibits high ($Na^+ + K^+ + Mg^{2+}$)-ATPase and adenylate cyclase activities. Additional activity of these enzymes is uncovered by sodium dodecyl sulfate and alamethicin, respectively. Electron microscopic inspection of the cell surface membrane preparation revealed (a) a predominance of thick-walled vesicles with smooth surfaces on negative staining and (b) binding of concanavalin A to the bulk of isolated membrane pieces following their incubation with the lectin.

Introduction

The crucial role of the cell surface membrane (cell membrane; sarcolemma in the muscle cell) in myocardial function is well recognized [1]. The myocardial cell membrane contains the channels for an ordered sequence of ion fluxes that form the basis for the action potential [2,3]. It is the seat of receptors for a variety of chemical signals which it transducts to the interior of the cell [2]. The biochemical analysis of these processes requires the characterization of the individual membrane components involved and depends on the availability of

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Abbreviations. EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulfate.

isolated cell surface membrane preparations of high purity.

A number of methods have been described for the mass isolation of pieces of cardiac cell surface membranes [4—18] *. The procedures usually involve homogenization followed by differential and/or density gradient centrifugation. Enrichment in cell surface membrane by these methods was documented by relatively high activities of marker enzymes for the plasma membrane [6,13—16] and by the recovery of cell surface membrane-bound ^{131}I [17]. However, contamination of the cell surface membrane fractions by fragments of sarcoplasmic reticulum cannot be excluded in view of the small differences in buoyant density of the two types of membrane [6].

Separation of different membrane types in heterogeneous membrane preparations can be improved by artificially enhancing existing differences in particle size and density. This approach has been successfully used in the past for the isolation of sarcoplasmic reticular membranes of cardiac and skeletal muscle. Vesicles derived from these membranes, which are distinguished by their ability to take up Ca^{2+} at a high rate in the presence of MgATP, were loaded with calcium oxalate or calcium phosphate, thus decisively increasing their buoyant density prior to their separation from other membrane types by sucrose density gradient centrifugation [19—22]. This method was improved by Levitsky et al. [23], who pointed out the importance of limiting the extent of loading for the isolation of the most active Ca^{2+}-accumulating vesicles.

In the present paper we demonstrate that partial loading of cardiac microsomes with calcium oxalate prior to their centrifugation in sucrose density gradients furnishes the basis, too, for an improved separation of membrane pieces derived from the cell surface. After selective sedimentation of the Ca^{2+}-loaded sarcoplasmic reticular membrane vesicles the unloaded, lightest microsomal subfraction, which is enriched in cell surface membrane fragments, is further purified by isopycnic centrifugation. In this way highly purified preparations of isolated cell surface membrane and sarcoplasmic reticulum were obtained from the same microsomal membrane source. The isolation method has proved to be useful in the study of protein kinase-catalyzed phosphorylation of cardiac membrane-bound proteins [24,25].

Materials and Methods

Materials. [α-^{32}P]ATP, specific activity 1—10 Ci/mmol, and $^{45}CaCl_2$, specific activity 10—40 Ci/g calcium, were purchased from The Radiochemical Centre, Amersham. Imidazole, L-histidine-HCl, and sodium cacodylate were obtained from Ferak, West Berlin. Sucrose (p.a.) and Dowex 50WX4 were from Serva, Heidelberg. Ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) was obtained from Schuchardt, München, and neutral alumina from Merck, Darmstadt. Cyclic AMP, cyclic GMP, ATP, GTP, phospho*enol*pyruvate, phosphocreatine, concanavalin A, pyruvate kinase, creatine phosphokinase, and horseradish peroxidase were products of Boehringer Mannheim GmbH. Disodium ATP was freed of sodium and neutralized with Tris by cation-

* Some of the authors cited [6,8—10,17,18] claim to have isolated the plasma membrane, although it seems quite likely, or even certain [10], that the basement membrane was not removed. Others [4,11, 12,15,16] call their isolated membrane fragments 'sarcolemma' but produce no evidence for the absence of substantial amounts of cell surface membranes from non-muscle cells.

exchange chromatography. Dithiothreitol was obtained from Calbiochem, Lubrol PX from Imperial Industries Ltd., and ouabain from VEB Berlin-Chemie. Alamethicin was the generous gift of Dr. G.B. Whitfield, Upjohn Company. For use a stock solution of 1.25 mg alamethicin/ml of 30% ethanol was prepared. All other substances were of analytical or reagent grade.

Fractionation of cardiac membranes. Cell surface and sarcoplasmic reticular membranes were purified from pigeon hearts in three succeeding steps: (1) preparation of microsomes; (2) loading of the microsomes with calcium oxalate followed by rate zonal centrifugation, and (3) isopycnic centrifugation of the lightest membrane fraction obtained in step (1). Step (1) and step (2) were performed as described by Levitsky et al. [23] with the modification that a smoother sucrose gradient was used in the rate zonal centrifugation step. Sucrose columns were prepared in centrifuge tubes of the Beckman SW 27 swinging bucket rotor by covering 2 ml of a 60% sucrose solution (w/v) containing 0.6 M KCl, 3 mM disodium ATP, 5 mM $MgCl_2$, 6.5 mM Tris/oxalate, 5 mM NaN_3, and 10 mM Tris-HCl buffer of pH 6.7 with a linear gradient formed from 10 ml of the same solution and 10 ml of 25% (w/v) sucrose solution containing 0.6 M KCl, 8 mM disodium ATP, 10 mM $MgCl_2$, 6.5 mM Tris/oxalate, 5 mM NaN_3, and 10 mM Tris-HCl buffer of pH 6.7. Centrifugation of calcium oxalate-loaded microsomes in these gradients for 3.5 h at 82 500 $\times g$ resulted in the separation of four distinct membrane fractions. The lightest fraction, which was recovered from the 20—28% sucrose layer, was purified further by isopycnic centrifugation. For this purpose membranes were diluted 3-fold with 0.6 M KCl and 3-ml aliquots of the suspension were layered onto linear density gradient columns prepared from 17.5 ml of 20% (w/v) and 17.5 ml of 33% (w/v) sucrose solutions. The gradients contained also 0.6 M KCl, 4 mM NaN_3, 5 mM disodium ATP, 5 mM $MgCl_2$, and 10 mM Tris-HCl buffer of pH 7.2. Centrifugation in a Beckman SW 27 swinging bucket rotor was then carried out for 16 h at 73 000 $\times g$. All membrane fractions were washed once with 0.6 M KCl and finally suspended in 40% (w/v) sucrose/10 mM Tris-HCl buffer of pH 7.2. They were kept on ice for immediate use or were stored at −70° C.

A scheme of the entire preparation procedure is presented in Fig. 1.

The specific density of the various sucrose layers was determined with a refractometer of Carl Zeiss Jena.

Assays. ATPase activity was determined at 37° C by measurement of inorganic phosphate split from ATP [26]. The reactions were usually carried out with 5—15 μg of membrane protein in a total volume of 250 μl. They were started after a 5 min preincubation by addition of Tris/ATP and terminated at 5 min (in the case of $(Ca^{2+} + Mg^{2+})$-ATPase) and at 10 min (for the other ATPases) by addition of 0.2 ml of ice-cold 0.4 M trichloroacetic acid.

$(Na^+ + K^+)$-stimulated ATPase activity was estimated as the difference of ATPase activity measured in 2 mM $MgCl_2$, 2 mM Tris/ATP, 100 mM NaCl, 10 mM KCl, 10 mM Tris-HCl buffer of pH 7.4 and ATPase activity measured in the same buffer, but without added NaCl and KCl. Ouabain-sensitive ATPase was the difference between ATPase activity in the absence and in the presence of 0.3 mM ouabain in a medium containing 2 mM $MgCl_2$, 2 mM Tris/ATP, 100 mM NaCl, 10 mM KCl, and 10 mM Tris-HCl of pH 7.4. Azide-sensitive

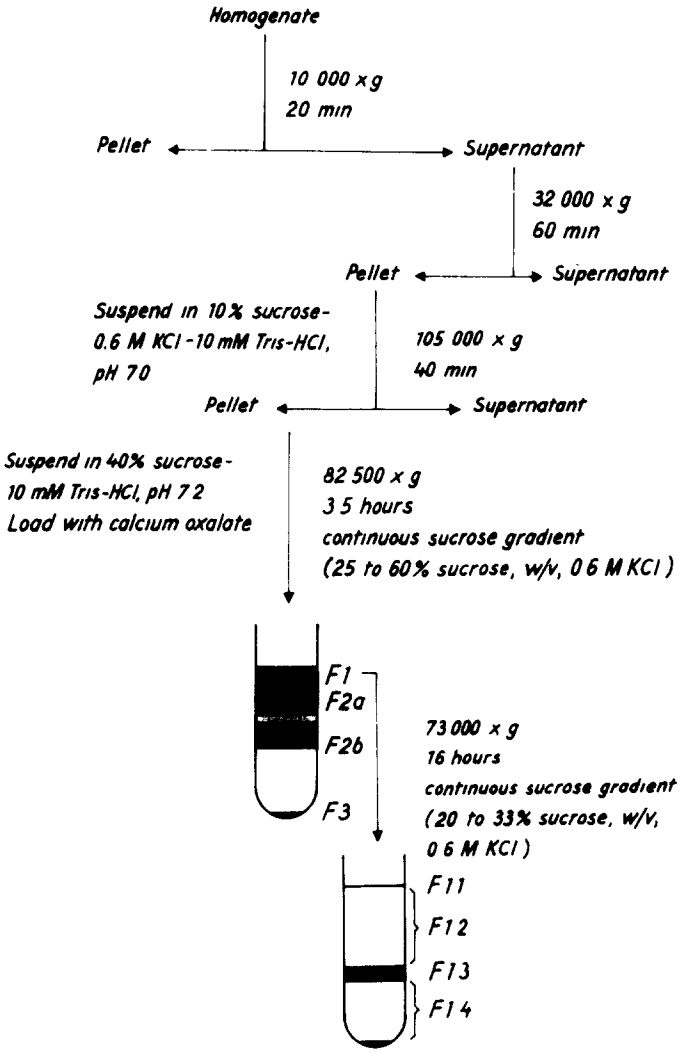


Fig. 1. Scheme for the isolation of sarcoplasmic reticulum and cell surface membranes from pigeon heart.

ATPase activity was taken as the difference in ATPase activity measured in the absence and in the presence of 5 mM NaN_3 in a solution containing 2 mM $MgCl_2$, 2 mM Tris/ATP, and 10 mM Tris-HCl buffer of pH 7.4. Ca^{2+}-activated ATPase was determined by subtracting ATPase activity measured in a medium consisting of 120 mM KCl, 5 mM $MgCl_2$, 5 mM Tris/ATP, 2.5 mM Tris/oxalate, 5 mM NaN_3, 1 mM EGTA, and 40 mM histidine-HCl buffer, pH 6.8, from ATPase activity measured in the same medium, but with EGTA replaced by 0.1 mM $CaCl_2$.

Pretreatment of membrane vesicles with SDS was carried out as described by Besch et al. [27].

Ca^{2+} uptake was measured as described by Martonosi and Feretos [28] at 25°C and with 10—15 μg of freshly isolated membrane protein in the presence

of 120 mM KCl, 5 mM $MgCl_2$, 5 mM Tris/ATP, 2.5 mM Tris/oxalate, 5 mM NaN_3, 1.45 mM EGTA, 1 mM $^{45}CaCl_2$ (specific activity: 20—50 Ci/mol), and 40 mM histidine-HCl buffer, pH 6.8. The total reaction volume was 1 ml.

Adenylate cyclase was measured at 37°C according to Ramachandran [29] in 40 mM Tris-HCl buffer of pH 7.5 containing, in a total volume of 150 µl 5.5 mM KCl, 15 mM $MgCl_2$, 4 mM aminophylline, 2 mM cyclic AMP, 7.2 mM phospho*enol*pyruvate, 8 mM NaF, 20 µg pyruvate kinase, 0.01% bovine serum albumin, and 0.4 mM [α-^{32}P]ATP (2 Ci/mol).

Protein was determined according to Lowry et al. [30] with ovalbumin as reference standard.

Concanavalin A binding experiments. Membrane samples containing about 5 mg protein were suspended in 20 ml of 0.1 M potassium phosphate buffer of pH 7.2 and sedimented for 30 min at 105 000 × *g*. The pellets obtained were fixed for 4—5 h at room temperature in a formaldehyde/glutaraldehyde fixative as specified by Karnovsky [31]. The fixed pellets were washed several times with 0.1 M phosphate buffer of pH 7.2 and carbohydrate groups were labeled with concanavalin A and horseradish peroxidase by the methods of Bernhard and Avrameas [32]. The catalytic activity of membrane-bound peroxidase was revealed with diaminobenzidine [31].

Electron microscopy. Fixation of membrane pellets and staining were performed by standard procedures [31,33]. Negative staining of the vesicle fractions was carried out with 2% ammonium molybdate that was adjusted to pH 7.2 [34]. The stained preparations were examined in a JEM 100B electron microscope.

Results

Fractionation of calcium oxalate-loaded cardiac microsomes

During rate zonal centrifugation of calcium oxalate-loaded pigeon heart microsomes in a sucrose density gradient (25—60% sucrose, w/v, and 0.6 M KCl) membrane vesicles filled with calcium salt sediment as a dense pellet at the bottom of the centrifuge tube. In addition, three fairly well separated membrane fractions can be recovered from the sucrose gradient: an upper yellow-white coloured layer, called fraction 1, at the 20/25% sucrose interphase, and below it two yellow-brown layers, designated here fractions 2a and 2b. The amount of protein which was recovered after collection of the fractions and after washing them with 0.6 M KCl constituted for membrane fractions 1, 2a, 2b, and 3, respectively, 7.7, 17.0, 11.0, and 4.9% of the total microsomal protein applied.

Membrane fraction 1 was found to be enriched in plasma membrane marker enzyme activities (see below); it was subjected to further purification by isopycnic centrifugation in a second density gradient (20—33% sucrose, w/v, and 0.6 M KCl). Two distinct fractions of differing specific density were separated from the sucrose columns: fraction 1.1, appearing as a white-coloured zone at the top of the gradient with an average density of 1.105 g/ml, and fraction 1.3, a yellow-white, sharply limited layer with a median density of 1.133 g/ml. The diffuse layer between fraction 1.1 and fraction 1.3 was harvested as fraction 1.2 and the lowest part of the sucrose column, including the pellet, was

collected as fraction 1.4. Expressed in per cent of total membrane protein of fraction 1 subjected to equilibrium centrifugation, the protein yield of subfractions 1.1, 1.2, 1.3, and 1.4, respectively, was 5.7, 23.1, 34.9, and 7.1%.

Enzyme activities of cardiac membrane fractions

($Na^+ + K^+ + Mg^{2+}$)-ATPase. In all cardiac membrane fractions tested in the present work, addition of 100 mM NaCl and 10 mM KCl caused a 2—4-fold stimulation of ATPase activity measured in the absence of the monovalent cations. The values for Na^+- and K^+-enhanced ATPase activity of cardiac microsomes and their fractions are listed in the first column of Table I.

To exclude Na^+- and K^+-stimulated activity of ($Ca^{2+} + Mg^{2+}$)-ATPase [35,36] EGTA was additionally included in the assays of fractions 1 and 3 and of subfractions 1.1—1.4 obtained by isopycnic centrifugation (Table I, second column). The values thus obtained represent only part of the total ($Na^+ + K^+ + Mg^{2+}$)-ATPase activity present in the various fractions. During homogenization of the myocardium both 'inside-in' ('right side-out') and 'inside-out' oriented membrane vesicles are formed along with leaky vesicles and open membrane fragments. Latent ($Na^+ + K^+$)-stimulated ATPase activity present in inside-in sarcolemmal vesicles and latent ouabain-sensitive ATPase activity of either inside-in or inside-out membrane vesicles can be expressed after disruption of the vesicles by detergents or in the presence of alamethicin, a channel-forming polypeptide [27,37]. In the present work ($Na^+ + K^+ + Mg^{2+}$)-ATPase activities were estimated according to Besch et al. [27], following preincubation of membrane fractions with SDS at a concentration of 0.3 mg/mg membrane protein and per ml of 40% sucrose/10 mM Tris-HCl buffer of pH 7.2. As was previously observed by Jones et al. [36], this pretreatment of the membrane resulted in minor losses of ($Na^+ + K^+ + Mg^{2+}$)-ATPase activity, but most of the ($Ca^{2+} + Mg^{2+}$)-ATPase disappeared.

Comparison of ouabain-sensitive ATPase activity before and after SDS treatment suggests that most of the cell surface membrane fragments present in KCl microsomes form tightly sealed vesicles. Ouabain-sensitive ATPase in SDS-treated KCl microsomes was more than 6 times higher than it was in untreated microsomes (Table I).

After rate zonal centrifugation in a sucrose gradient ouabain-inhibited ATPase activity was enriched in membrane fraction 1 (Table I). Without preceding detergent treatment the specific enzyme activity in the latter membranes was increased 13-fold over that in the KCl microsomes. The increase was only 4-fold when the SDS-treated membranes are compared. The data indicate, that a considerable part of the cell surface membranes enriched in fraction 1 forms open fragments or leaky vesicles. Further enrichment of ouabain-sensitive ATPase activity is achieved by isopycnic centrifugation of membrane fraction 1 (Table I). Among the four fractions collected after equilibrium centrifugation, the highest activity was found in the lightest membrane fraction 1.1, the second and the third highest in fractions 1.2 and 1.3, respectively. Differences observed between untreated and SDS-treated membrane fractions 1.1—1.3 with respect to ouabain-sensitive ATPase activities are small, indicating that leakiness of cell membrane vesicles in these membrane fractions was further increased during their preparation from fraction 1.

TABLE I

DISTRIBUTION OF Na^+- AND K^+-STIMULATED ATPase ACTIVITY IN PIGEON HEART MICROSOMES AND THEIR SUBFRACTIONS

Fractions 1, 2a, 2b, and 3 were obtained by rate zonal centrifugation of calcium oxalate-loaded microsomes in a 25—60% sucrose (w/v) gradient containing 0.6 M KCl. Fractions 1.1—1.4 were obtained from fraction 1 by equilibrium centrifugation in a second linear density gradient prepared from 20 and 33% sucrose (w/v) in 0.6 M KCl. Enzyme activities were measured at 37°C as described in Materials and Methods after a 20 min preincubation of the membranes at room temperature in the absence (untreated membranes) or presence of 0.3 mg SDS/ml per mg membrane protein (SDS-treated membranes). Activity values represent means ± S.E. calculated from duplicate measurements on 4—6 individual membrane preparations. Recovery of enzyme activity in fractions 1, 2a, 2b, and 3 is given in parentheses as per cent of total enzyme activity of KCl microsomes. Recovery of enzyme activity in fraction 1.1—1.4 is given in per cent of enzyme activity of fraction 1. n.d., not determined.

Membrane fraction	$(Na^+ + K^+)$-stimulated ATPase (nmol P_i/mg protein per min)				Ouabain-sensitive ATPase (nmol P_i/mg protein per min)	
	Untreated membranes		SDS-treated membranes			
	—EGTA	+EGTA	—EGTA	+EGTA	Untreated membranes	SDS-treated membranes
KCl microsomes	202 ± 23 (100)	n.d.	145 ± 23 (100)	n.d.	12 ± 5 (100)	75 ± 25 (100)
Rate zonal centrifugation:						
1	358 ± 30 (14)	273 ± 33	378 ± 28 (19)	372 ± 37	158 ± 13 (102)	272 ± 20 (28)
2a	347 ± 20 (29)	n.d.	161 ± 33 (19)	n.d.	35 ± 5 (50)	102 ± 27 (23)
2b	257 ± 15 (13)	n.d.	99 ± 12 (8)	n.d.	5 ± 5 (5)	65 ± 23 (10)
3	310 ± 35 (8)	50 ± 30	145 ± 23 (5)	23 ± 12	3 ± 3 (1)	25 ± 10 (2)
Isopycnic centrifugation:						
1.1	460 ± 53 (7)	372 ± 48 (8)	385 ± 40 (6)	n.d.	315 ± 26 (11)	340 ± 55 (7)
1.2	347 ± 38 (22)	243 ± 8 (22)	286 ± 23 (18)	n.d.	213 ± 15 (31)	223 ± 12 (19)
1.3	275 ± 15 (25)	203 ± 23 (28)	198 ± 24 (18)	n.d.	200 ± 25 (44)	188 ± 22 (24)
1.4	310 ± 35 (6)	110 ± 23 (3)	142 ± 18 (3)	n.d.	40 ± 9 (2)	100 ± 18 (3)

Lowest ouabain-sensitive ATPase activity was found in membrane fraction 3, obtained in the first rate zonal centrifugation step (Table I). This fraction exhibits only negligible activity without preceding detergent treatment, but some activity becomes measurable following preincubation with SDS. The few cell surface membrane fragments in fraction 3 appear thus to be tightly sealed. As judged from the relatively high activity of Na^+- and K^+-stimulated ATPase of the untreated fraction 3 membranes in the presence of EGTA, sealed cell membrane vesicles in fraction 3 possess predominantly an inside-out orientation. It can be assumed that K^+ were present inside intact inside-out vesicles, since both the final step in the preparation of the microsomes and the gradient centrifugations were performed in the presence of 0.6 M KCl and since K^+ permeability of the cardiac cell membrane is relatively high.

(Ca^{2+} + Mg^{2+})-ATPase. Sodium azide-insensitive (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} uptake activities, reckoned per mg membrane protein, were 3 and 12 times higher, respectively, in the sedimented fraction 3 than they were in KCl microsomes. As much as 61.5% of the total microsomal Ca^{2+} uptake activity was recovered in fraction 3 (Table II). But Ca^{2+}-dependent ATPase and Ca^{2+} transport activities were also encountered in all other membrane fractions obtained on rate zonal and isopycnic centrifugations.

Although Ca^{2+} uptake and Ca^{2+}-dependent ATPase were not measured under identical conditions, the ratio of the former to the latter may still be taken as a rough index of the coupling efficiency of the two processes. In the most active Ca^{2+}-accumulating fraction, membrane fraction 3, this ratio was 4 times higher

TABLE II

(Ca^{2+} + Mg^{2+})-ATPase, Ca^{2+}-UPTAKE RATE, AND AZIDE-SENSITIVE ATPase IN PIGEON HEART MICROSOMES AND THEIR SUBFRACTIONS

(Ca^{2+} + Mg^{2+})-ATPase was measured at 37°C in the presence of 5 mM NaN_3, as described in Materials and Methods. Ca^{2+} uptake rate was calculated from the amount of Ca^{2+} accumulated by the membrane in a 2 min interval at 25°C in the presence of 5 mM NaN_3, 5 mM Tris/oxalate, 1 mM $CaCl_2$, and 1.45 mM EGTA. Azide-sensitive ATPase was estimated at 37°C as the difference between ATPase activity in the presence and in the absence of 5 mM NaN_3. Activity values represent means ± S.E. of estimates in 4—6 individual preparations. Recovery of enzyme and Ca^{2+} uptake activities is indicated in the parentheses as percentage of the corresponding value in the KCl microsomes. For details of membrane separation and reference values for enzyme and Ca^{2+} uptake activities see also Table I.

Membrane fraction	(Ca^{2+} + Mg^{2+})-ATPase (nmol P_i/mg protein per min)	Ca^{2+} uptake (nmol Ca^{2+}/mg protein per min)	Azide-sensitive ATPase (nmol P_i/mg protein per min)
KCl microsomes	832 ± 48 (100)	150.6 ± 30 (100)	700 ± 81 (100)
Rate zonal centrifugation:			
1	488 ± 40 (5)	46.0 ± 3.4 (24)	68 ± 18 (1)
2a	389 ± 53 (8)	27.4 ± 3.1 (3.1)	355 ± 53 (9)
2b	224 ± 42 (3)	19.6 ± 5.1 (1.4)	442 ± 15 (7)
3	2627 ± 118 (16)	1878 ± 375 (61.5)	248 ± 30 (2)
Isopycnic centrifugation:			
1.1	437 ± 47 (5)	43.0 ± 4.6 (5)	22 ± 10 (2)
1.2	420 ± 38 (20)	29.8 ± 1.7 (15)	28 ± 22 (10)
1.3	181 ± 27 (13)	18.0 ± 1.8 (14)	37 ± 15 (19)
1.4	500 ± 72 (7)	59.7 ± 2.0 (9)	108 ± 42 (11)

than it was in the KCl microsomes, presumably because of the inside-in orientation of tightly sealed sarcoplasmic reticulum membrane vesicles in this fraction, i.e. their cytoplasmic face turned to the outside. This allows for Ca^{2+} accumulation inside the vesicles. All other fractions recovered from the sucrose gradients exhibited lower ratios of Ca^{2+} uptake to Ca^{2+}-dependent ATPase activity.

Azide-sensitive ATPase, which is an attribute of mitochondria [38], was highest in membrane fractions 2a and 2b of the first sucrose gradient. Only low activities of this enzyme have been found in fraction 1 and its subfractions 1.1—1.3 (Table II).

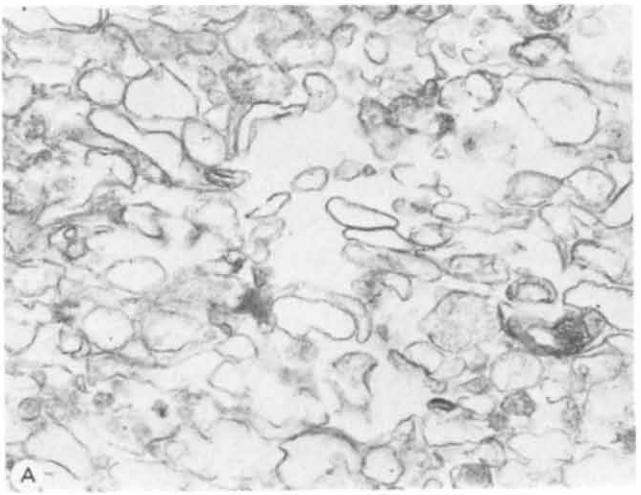
Adenylate cyclase. Specific activities of basal and NaF-stimulated adenylate cyclase in membrane fraction 1 were 2—3 times higher than they were in the KCl microsomes (Table III). Fraction 3 contained only 1/13 of the activity present in fraction 1. NaF-stimulated activity in both membrane fractions 1 and 3 is enhanced further by about 30% in the presence of 1 mM EGTA (data not shown). This increase is presumably due to complexation of Ca^{2+} which inhibits cardiac adenylate cyclase [39]. Additional adenylate cyclase activity is uncovered in fraction 1 by the antibiotic alamethicin (Table III, third column). As was suggested by Besch et al. [37] channels formed through vesicle membranes by alamethicin are of sufficient size to allow entry of ATP and NaF, thus eliminating the influence of membrane sidedness on adenylate cyclase activity associated with the membrane vesicles. The observed effect of alamethicin indicates that nearly half of the membrane material in fraction 1 consists of sealed inside-in oriented vesicles.

TABLE III

ADENYLATE CYCLASE ACTIVITY IN CARDIAC MICROSOMES AND SUBFRACTIONS OBTAINED BY RATE ZONAL AND ISOPYCNIC CENTRIFUGATION OF CALCIUM OXALATE-LOADED MICROSOMES

Adenylate cyclase activity was estimated at 37°C in the absence (basal) and presence of 8 mM NaF. Alamethicin was added in some preparations in a final concentration of 0.16 mg/ml and per 0.165 mg protein. This concentration was maximally effective. Means ± S.E. of activity estimates in 3—6 membrane preparations. Enzyme recovery is given in parentheses in per cent of total enzyme activity of KCl microsomes (fractions 1, 2a, 2b, and 3) and fraction 1 (fractions 1.1—1.4).

Membrane fraction	Adenylate cyclase activity (nmol cyclic AMP/mg protein per min)		
	Basal	NaF stimulated	NaF and alamethicin stimulated
KCl microsomes	0.194 ± 0.036 (100)	0.478 ± 0.106 (100)	
Rate zonal centrifugation:			
1	0.541 ± 0.085 (21)	1.105 ± 0.189 (18)	2.00 ± 0.15
2a	0.183 ± 0.055 (16)	0.456 ± 0.107 (16)	
2b	0.083 ± 0.042 (5)	0.176 ± 0.052 (4)	
3	0.040 ± 0.014 (1)	0.087 ± 0.025 (1)	0.12 ± 0.03
Isopycnic centrifugation:			
1.1	0.692 ± 0.041 (7)	1.200 ± 0.112 (6)	
1.2	0.507 ± 0.023 (22)	1.066 ± 0.084 (22)	
1.3	0.434 ± 0.043 (28)	0.770 ± 0.079 (24)	
1.4	0.092 ± 0.045 (1)	0.241 ± 0.06 (2)	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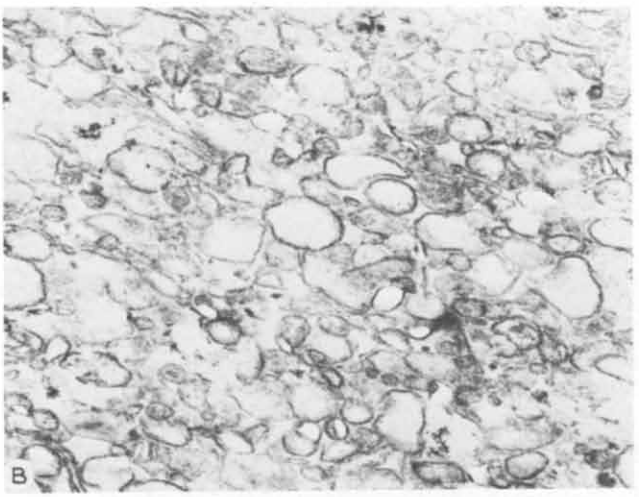
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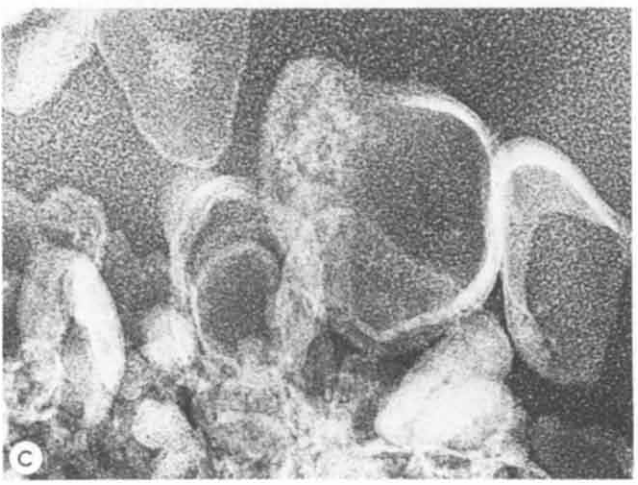
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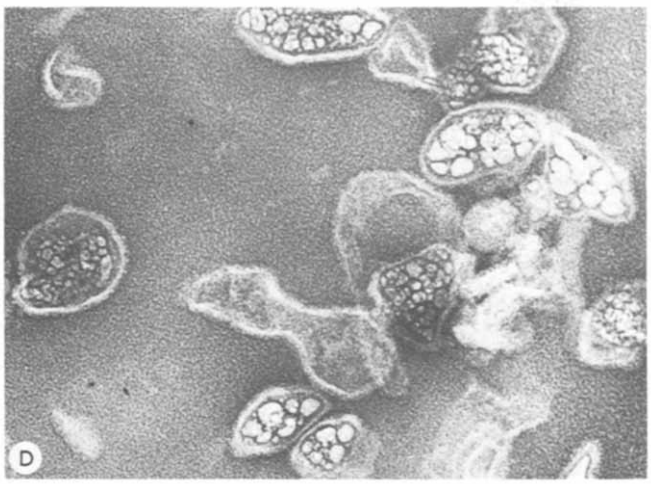


Fig. 2. Transmission electron micrographs of membrane fraction 1.1 and membrane fraction 3. Thin sections of positively stained membrane fraction 1.1 (A) and membrane fraction 3 (B). ×48 000. Negatively stained membrane fraction 1.1 (C) and membrane fraction 3 (D). ×114 200.

After isopycnic centrifugation highest adenylate cyclase activity was present in the lightest subfractions 1.1 and 1.2, with lower activities in the other fractions. The distribution of adenylate cyclase in the various membrane fractions thus resembles the distribution of $(Na^+ + K^+ + Mg^{2+})$-ATPase activity and contrasts to that of Ca^{2+}-dependent ATPase and Ca^{2+} transport activity.

It is of considerable interest that the sarcoplasmic reticular fraction 3, which is poor in adenylate cyclase, exhibits an activity of guanylate cyclase as high as 0.67 nmol cyclic GMP/mg protein per min [40]. This activity is even higher than that found in cell surface membrane fraction 1.1, where it amounted to about 0.25 nmol cyclic GMP/mg protein per min.

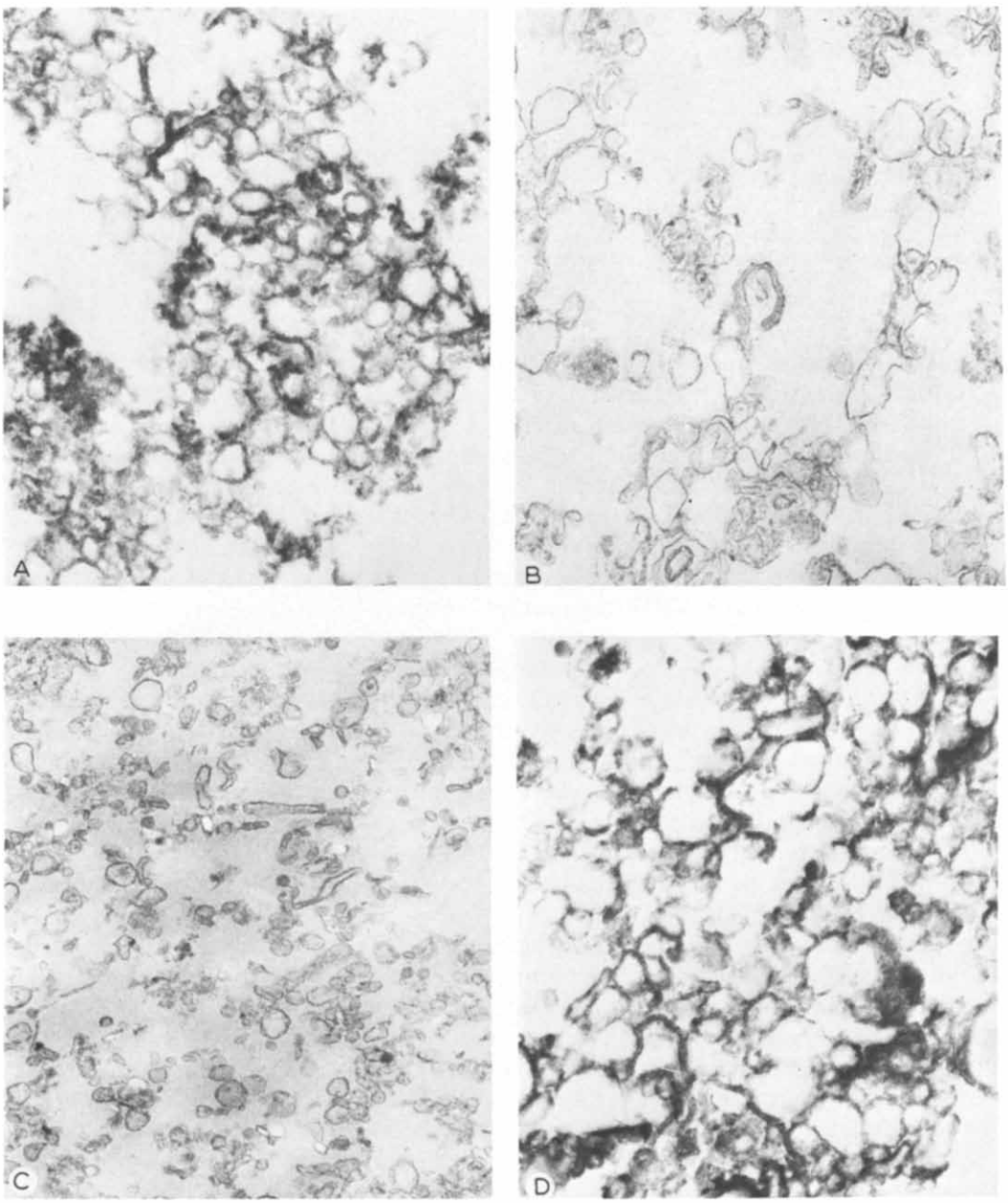
Ultrastructural characterization of cardiac membrane fractions

Negative staining. After negative staining thick-walled membrane vesicles with smooth surfaces were observed in fraction 1.1 (Fig. 2C). They were also found in fraction 1 and subfractions 1.2 and 1.3 (not shown). Vesicle surfaces in fraction 3 (Fig. 2D), on the contrary, appear rough and covered with knob-like particles similar to the 4-nm particles observed in isolated skeletal muscle sarcoplasmic reticulum [41]. About 80% of these fraction 3 membrane vesicles contain deposits of calcium oxalate, as revealed by systematic evaluation of electron micrographs.

Concanavalin A binding. Heavy accumulation of concanavalin A is seen in the cell surface-enriched fraction 1 and, even more so, in its subfractions 1.1—1.3 (Fig. 1A, D, E). Specificity of concanavalin A binding was demonstrated in control experiments carried out in the presence of 0.1 M α-methyl-D-mannoside. The latter prevented lectin binding completely (Fig. 3B). Staining appears to be especially prominent in a limited area of some vesicles and in open membrane fragments. In contrast to the cell surface-derived membranes the sarcoplasmic reticular membrane vesicles contained in fraction 3 do not bind concanavalin A (Fig. 3C).

Discussion

Rate zonal and isopycnic centrifugation of calcium oxalate-loaded cardiac microsomes was employed for the fractionation of pigeon heart membranes. Pigeon hearts were used because, like hearts of other avian species, they lack the T-system [42], which consists of tubular invaginations of the sarcolemma



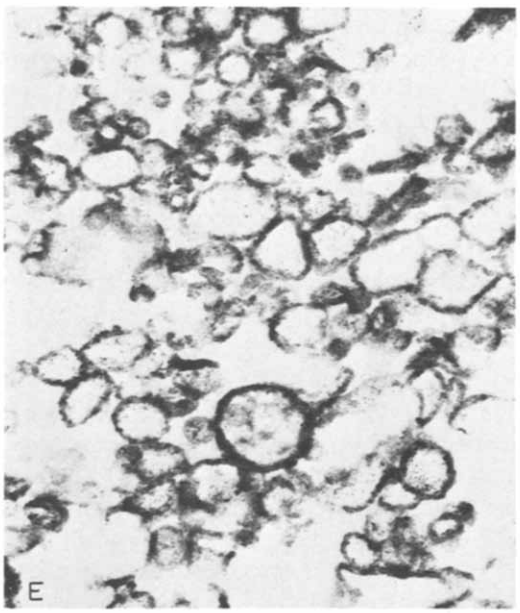


Fig. 3. Binding of concanavalin A to cardiac cell surface membranes. Concanavalin A binding was revealed by the diaminobenzidine reaction after coupling of horseradish peroxidase to the membrane-bound lectin. ×48 000. (A) Fraction 1; (B) fraction 1 in the presence of 0.1 M α-methyl-D-mannoside; (C) fraction 3; (D) fraction 1.1, and (E) fraction 1.3.

that invade deeply into the mammalian heart cell [2]. The separation of T-tubules from the terminal sacs of the sarcoplasmic reticulum requires special procedures (see Ref. 43). A major advantage of the present isolation procedure is that it allows the parallel purification of cell surface and sarcoplasmic reticular membranes from the same microsomal membrane source. The easily sedimenting membrane fraction, which is the one most active in Ca^{2+} transport, is derived mainly from the sarcoplasmic reticulum [23]. In contrast, the lightest membrane fractions (fractions 1 and, to a higher degree, its subfractions 1.1 and 1.2) are enriched in vesicles and other fragments originating from the cell surface membrane, as suggested by three lines of evidence:

(1) The buoyant density of subfractions 1.1 and 1.2 is 1.105 and 1.120 g/ml, respectively. These low density values are close to density values reported previously for cell membrane preparations of cardiac muscle [6,17]. The isolation of cardiac sarcolemmal membrane pieces of apparently higher density (they sediment in 50% sucrose) was reported by Hui et al. [13] and St. Louis and Sulakhe [14]. As pointed out by Heller and Harary [17], changes in apparent membrane density occur easily during freezing and thawing, at high salt concentrations, and in cell homogenates.

(2) The cell surface membrane fractions (fraction 1 and subfractions 1.1 and 1.2) exhibit high adenylate cyclase and $(Na^+ + K^+ + Mg^{2+})$-ATPase activities. Whereas $(Na^+ + K^+ + Mg^{2+})$-ATPase is an accepted marker enzyme for cell surface membranes [44,45], there is some uncertainty concerning the cellular distribution of adenylate cyclase in heart muscle. Adenylate cyclase has been

found to be associated with the plasma membranes of most animal cells [44] and quantitative biochemical as well as cytochemical investigations have shown that in heart muscle the enzyme resides predominantly in the cell surface [11,13,14,37,39,46—48]. However, adenylate cyclase activity has been claimed to be present also in cardiac sarcoplasmic reticulum [49,50]. It appears from the present data that adenylate cyclase copurifies with $(Na^+ + K^+ + Mg^{2+})$-ATPase in the light membrane fractions and, as pointed out above, that there is in all likelihood only negligible adenylate cyclase activity associated with the most active Ca^{2+}-transporting vesicles, which are derived from the sarcoplasmic reticulum. However, we cannot exclude the presence of the enzyme in the terminal cisternae [48,51].

(3) Most membrane vesicles present in the cell membrane fraction 1 and the bulk of the vesicles in fractions 1.1—1.3 bind concanavalin A (Fig. 3). Concanavalin A has been shown to bind specifically to α-D-mannosides and α-D-glycosides and has been used to locate these carbohydrates on cell surface membranes [32,52—54]. In experiments with both whole skeletal muscle and various membrane fractions of skeletal muscle concanavalin A bound also predominantly to the sarcolemma, with no binding to sarcoplasmic reticulum and mitochondria [54]. It should be emphasized that in the present study incubation with concanavalin A was carried out for only 30 min. This may be too short an incubation time to allow for penetration of concanavalin A into closed membrane vesicles [32]. This would mean that the stained vesicles, which represent the majority of vesicles in the cell surface membrane fractions, are leaky and/or have an inside-in orientation (see also Results).

A question requiring scrutiny is whether or not Ca^{2+}-activated Mg^{2+}-ATPase, which is the major protein in sarcoplasmic reticulum [23], is present also in cardiac sarcolemma, as has recently been claimed [13,14]. According to Barchi et al. [54] $(Ca^{2+} + Mg^{2+})$-ATPase is a constituent of skeletal muscle sarcolemma. The present data are interpretable as being compatible with the existence of a cardiac sarcolemmal $(Ca^{2+} + Mg^{2+})$-ATPase. After effective removal of the most active Ca^{2+}-accumulating membrane vesicles by centrifugation of calcium oxalate-loaded microsomes, substantial activity of Ca^{2+}-dependent Mg^{2+}-ATPase and slight Ca^{2+} uptake were found in the plasma membrane-enriched fraction 1 and its subfractions 1.1 and 1.2. Inside-out oriented vesicles of sarcoplasmic reticular membranes, which might be present in these fractions (see also Ref. 55), would not be expected to contribute to the Ca^{2+}-dependent activities. Assuming, furthermore, that $(Na^+ + K^+ + Mg^{2+})$-ATPase is located in pigeon heart muscle exclusively in the plasma membranes, the presence of some $(Na^+ + K^+ + Mg^{2+})$-ATPase activity in fraction 3, which consists of calcium oxalate-loaded vesicles, could be indicative of the presence of $(Ca^{2+} + Mg^{2+})$-ATPase in those membranes. Only those cell surface membrane vesicles are expected to sediment in 60% sucrose/0.6 M KCl which have accumulated sufficient amounts of calcium oxalate. Cell surface membrane vesicles exhibiting ATP-dependent Ca^{2+} accumulation can be assumed to have an inside-out orientation (see Results). Further study of Ca^{2+} transport in cardiac cell surface membranes may reveal more of its basic features.

Acknowledgements

We thank Christel Kemsies, Evelyn Bolick, and Irmgard Küttner for excellent technical assistance.

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