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PLASMA MEMBRANES FROM CARDIAC CELLS IN CULTURE

ENZYMATIC RADIO-IODINATION, EVALUATION OF PREPARATION AND PROPERTIES OF THE SARCOLEMMA

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Summary

Plasma membranes from heart (sarcolemma) were prepared by the method of Kidwai, A.M. ((1975) Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. XXXIA, pp. 134—144, Academic Press, New York). On many occasions the sarcolemmal fraction identified by the enzyme markers such as (Na^+ + K^+)-ATPase banded at heavier densities ($d > 1.25$ g/ml) than expected for plasma membrane ($d < 1.15$ g/ml). Radio-iodination of the membrane was added as an independent marker and conditions for the reproducible preparation of the sarcolemma were studied. Cultured heart cells were enzymatically iodinated under conditions which did not affect viability and labeled primarily the sarcolemma. The distribution of radioactivity in homogenates of cultured cells on the density gradient corresponded to that of the enzymes' activity. The best sarcolemma preparation was obtained with 0.3 M KCl extraction of heart homogenates in the presence of 0.05 M pyrophosphate, especially if the salt was also present during the fractionation by density gradient centrifugation. Alterations in the density were also observed with erythrocytes and cultured liver cells' plasma membrane. The data suggests a meta-stable state of the plasma membranes due to handling or storage which could cause alterations of some of their physical properties (e.g. density).

Introduction

The complexity and the toughness of the cardiac muscle sarcolemma and the cells' complex internal structure intimately associated with the sarcolemma has hampered its easy isolation. Confusion exists as to the appropriate method of

homogenization or to the choice of the mode of separation of the subcellular components despite attempts to clarify the situation [2].

Most reports on the preparation of sarcolemma present the method with little background information [3,4] making it difficult to evaluate the method or to adapt it. In addition, various contradictory techniques have been reported. Some indicate that the sarcolemma bands at densities at or below d = 1.15 g/ml and others report it to band at a value higher than 1.25 g/ml [5,6]. Reports on the stability of the membranes and the enzyme content have also varied. Very little data has been presented as to yield, purity and physical properties of these preparations. Such studies could give a rational basis for the preparation and storage of the sarcolemma.

Our purpose has been to prepare heart cell plasma membranes from cultured heart cells using enzyme and radioactive markers, comparing several homogenization techniques, homogenization media, storage methods and means of separation to give a rational basis for membrane preparation. In addition we have attempted to get some idea of the yield, purity and of the physical properties of the sarcolemma. Our results indicate changes in the sarcolemmal properties which could occur during the preparation or storage suggesting an unstable structure which could explain the conflicting and inconsistent data in the literature.

Materials

CMRL-1415A prepared according to Healy and Parker [7], was sterilized by ultrafiltration and stored at 4°C. Serum solutions from horse and calf were purchased from GIBCO (Grand Island Biological Co., Grand Island, N.Y.). Tyrode solution was prepared according to Tyrode [8]. *p*-Nitrophenyl phosphate, Tris salt, cytochrome *c*, reduced with $Na_2S_2O_4$ or ascorbate [9,10], Na_2 ATP, lactoperoxidase, EC 1.11.1.7 (Lots 95 c 0068 and 36 c 9560); glucose oxidase, EC 1.1.3.4 (lot 45 c 0062-1) were Sigma products (Sigma Co., St. Louis, Mo.). Ficoll 400 was purchased from Pharmacia (Uppsala, Sweden). Viokase, a pancreatic extract containing proteases, was from Viobin Corp. Monticello, Ill.). Sepharose-immobilized lactoperoxidase, EC 1.11.1.7 (lot 95A003) was purchased from Worthington Biochemical Corp. (Freehold, N.J.). Sucrose for density gradient (or enzyme grade) was a Schwartz-Mann product (Orangeburg, N.Y.). Preparation of fluorescent anti-myosin antibodies was based on the methods of Palmitter et al. [11] and Johnson and Holborow [12] as outlined in ref. 13.

Methods

Hearts were obtained from adult (approx. 150 g) or 0—4-days-old rats. Cell cultures were prepared using two neonatal hearts/petri dish 100 mm in diameter [14] in a chemically defined medium CMRL - 1415A [7] containing 10% horse and calf sera (CMRL - S). Confluency was reached after 3—4 days at 37°C. Enrichment of muscle cells was achieved by preplating for 45 or 90 min causing selective attachment of non-muscle cells [15,16]. Monolayer cultures of rat liver cells (line RLC-GAI) were grown according to Gerschenson et al.

[17] and kindly donated by Dr. L. Lake. Human outdated blood in citrated dextrose was obtained from the Los Angeles Red Cross Blood Bank.

Homogenization and fractionation. Intact heart tissue or cultured cells were homogenized and fractionated according to Kidwai [1] using Brinkmann Polythron Homogenizer PT-10-35 (Brinkmann Instruments, Westbury, N.Y.) at half maximal speed for 20 s. The homogenate or pellets obtained after centrifugation for 60 min at $10^5 \times g$ were resuspended and fractionated by density gradient centrifugation. Aliquots of approx. 2 ml were layered on a 17 ml or 34 ml tube containing continuous 8—54 or 33—54% sucrose gradients. The tubes were spun in a SW27 rotor, Spinco L-50 ultracentrifuge (Beckman, Palo Alto), for 90—300 min at 27 000 rev./min (110 000 × *g*). Fractions of 2—4 ml were collected from the top of the tube by means of a peristaltic pump (Auto Densiflow, IIc, Buchler, Instruments, Searle Analytic Inc., Fort Lee, N.Y.) and a fraction collector. Analysis was done immediately or else the fractions were stored at −20°C.

Analyses, assays and other determinations. Protein content was determined with specific modification for membrane proteins [18,19]. K^+-activated, ouabain-inhibited, phosphatase activity was determined with *p*-nitrophenyl phosphate as substrate, and a final concentration of 10 mM ouabain, added to the enzyme prior to the substrate and KCl, incubated for 10 min at 37°C [20]. Occasionally ($Na^+ + K^+$)-ATPase was assayed [5]. Cytochrome *c* oxidase activity was measured with reduced cytochrome *c* [9,10], Ca^{2+}-activated myosin ATPase was assayed after extraction with KCl [21]. The density of the fractions from the gradient (sucrose of Ficoll solutions) was measured with a Spencer 1717 (U.S.) refractometer. Cells were counted either before plating or after reaching confluency and release from the plate by proteolysis using 0.25% Viokase and 0.025% EDTA in ($Ca^{2+} + Mg^{2+}$)-free phosphate-buffered saline, (phosphate-buffered saline A) incubated at 37°C for 10 min. The cell suspension was diluted 1 : 10 or 1 : 20 with phosphate-buffered saline A and counted in a particle counter (Electrozone/Celloscope, 100 series, Particle Data Inc., Elmhurst, Ill.).

The myosin-containing cells were determined by the fluorescent anti-myosin antibody method [13]. For electron microscopy of cells or membranes, pellets were fixed by 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h, rinsed in 0.2 M buffer, fixed for 1 h in 1% OsO_4, dehydrated in graded series of alcohols and embedded in Epon. Sections were cut in Porter-Blum microtome MT-2, counter stained with lead nitrate and uranyl acetate and viewed with a Siemens 1A microscope. Lipids were extracted from cell homogenates and fractions from the gradient. Aliquots of the upper, aqueous-methanolic and lower, chloroform, phases were withdrawn for counting with correction for quenching by chloroform [22,23].

Enzymatic radio-iodination of cells and determination of radioactivity. The iodination procedure was adapted from several sources [24—26]: A monolayer culture (heart or liver) was rinsed with tyrode G (tyrode solution containing 15 mM glucose), and the following compounds were added in 10 ml tyrode G with final concentration indicated: KI, $2 \cdot 10^{-6}$—$8 \cdot 10^{-6}$ M; ^{131}I or ^{125}I, 5—50 μCi/plate; lactoperoxidase; soluble, 300—800 munits/ml; Sepharose-immobilized lactoperoxidase, 70 mI.U./ml. Glucose oxidase, 67 munits/ml.

After incubation at room temperature (10—30 min) the plates were placed on ice, decanted and ice-cold CMRL-S added. After several careful rinsings, the cells were homogenized and fractionated. Each fraction was counted in a gamma counter (Biogamma, Beckman). The counting efficiency was determined with ^{137}Cs to be about 42%. No quenching of the radioactivity of ^{125}I was detected using aqueous or methanolic solutions. However, chloroform or chloroform and methanol at 2 : 1 (v/v) inhibited the rate of counting by 37 and 49%, respectively. The chloroform phase containing lipids counted under these circumstances was corrected by a factor of 1.6. (cf. ref. 27).

Results

K^+-activated phosphatase of cardiac membranes

Rat heart homogenates, prepared and fractionated by sucrose density gradient centrifugation according to the method of Kidwai [1], showed a wide distribution of the K^+-activated, ouabain-inhibited phosphatase (K^+-*p*-nitrophenyl phosphatase). This enzyme is believed to be associated with the plasma membranes as well as the ($Na^+ + K^+$)-activated ATPase, which showed a similar pattern. Only 25% of the phosphatase activity was associated with a cell fraction banding at density considered to be enriched with plasma membranes or cardiac sarcolemma (e.g. $d < 1.159$ g/ml) [5] (Table IA). Most of the activity banded at heavier densities (e.g. $d > 1.254$ g/ml) where other subcellular com-

TABLE I

FRACTIONATION OF HEART HOMOGENATES FROM NEONATAL RATS BY SUCROSE DENSITY CENTRIFUGATION

Hearts homogenized by Polythron in 0.25 M sucrose, were centrifuged for 60 min at $10^5 \times g$, and the pellets layered on a continuous sucrose gradient (8—54%), centrifuged at $10^5 \times g$ for 90 min using rotor No. SW 27. Fractions were collected from the top, and their refractive index, protein content and K^+-activated phosphatase were determined. Expt. A: Fractionation of the $10^5 \times g$ pellet from the homogenate (fractions F_1—F_9). Expt. B: Refractionation of the frozen and thawed dense fractions F_8 and F_9 of Expt. A, on a fresh sucrose gradient (designated F_8^* and F_9^*, respectively; and the "top" and "bottom" (1—2) and (6), respectively). 100% activity was taken as the value prior to the recentrifugation.

Expt.	Fraction	Protein		K^+-activated phosphatase			Density (g/ml)	Sucrose (%)
		mg	%	munits	%	munits/mg protein		
A	$10^5 \times g$ pellets			1860				
	Total F_1—F_9	33		2260		69		
	Total F_1—F_6		13	563	25	139	<1.159	8—35
	F_7		5	90	4	60	<1.198	36—43
	F_8		19	384	17	61	<1.254	43—54
	F_9		63	1223	54	58	>1.254	54
B	F_8^* (1—2)				14		<1.1612	37
	(6)				61		<1.2296	50
	F_9^* (1—2)				18		<1.1612	37
	(6)				62		>1.2406	52

ponents are expected to be. A 2-fold purification of the phosphatase was obtained in the light fraction. Dilution, rehomogenization, and recentrifugation of the dense fraction on a fresh gradient, brought only 10—14% up to the lighter density range (i.e. d = 1.16 g/ml). More than 60% of the applied enzyme, banded at its original density range (Table IB); the rest was smeared over the gradient. This pattern of distribution was frequently observed with either intact hearts of adult and neonatal rats or cultured cardiac cells from neonatal rats. However, on several occasions, the phosphatase distributed differently with more enzymatic activity banded at the lighter part of the gradient (e.g. $d < 1.16$ g/ml). A possible effect of sucrose on the enzyme distribution was excluded by the similarity of banding on either sucrose or Ficoll 400 (Table II).

When either the low or high density fractions were each separately (both with approx. 280 munits of the enzyme) refractionated on a fresh sucrose gradient, 71% of the low and 93% of the high density fraction banded in the high density region on the new gradient (e.g. d = 1.219 g/ml). These results demonstrate a change in the density of the membranes and may be the cause of the variability observed in the distribution of the membranes carrying the phosphatase, assuming, of course, that this K^+-activated phosphatase is a legitimate sarcolemmal marker.

In order to test whether our procedures did not affect the integrity of other subcellular particles and upset the banding pattern, the distribution of both the phosphatase and cytochrome c oxidase was determined. The oxidase is a typi-

TABLE II

FRACTIONATION OF HEART CELL HOMOGENATES ON DENSITY GRADIENTS OF SUCROSE OR FICOLL

The details of the experiment are similar to those of Table I, with cultured heart cells from neonatal rats. Approx. 1/3 and 2/3 of the $10^5 \times g$ pellets were fractionated on sucrose and Ficoll, respectively.

Fraction	Density (g/ml)	Sucrose or Ficoll (%)	Protein (mg)	K^+-*p*-nitrophenyl phosphatase activity	
				munits	munits/mg
Homogenate			39.3	192	5
$1.5 \cdot 10^5 \times g$					
supernatant			5.4	0	0
pellet			26.1	632	24
Sucrose					
I	1.064	16	0.30	171 { 5	16
II	1.151	35	2.88	171 { 166	58
III	1.213	47	2.23	29	13
IV	>1.235	>51	5.95	90	15
Ficoll-400					
I	1.034	10	0.50	125 { 27	54
II	1.1036	30	3.13	125 { 98	31
III	1.127	36.5	1.57	7	5
IV	1.182	>54.5	13.49	113	8

cal mitochondrial enzyme and it banded predominantly (71%), as expected, at the center of the gradient. The top and bottom zones (low and high densities) contained 26 and 3%, respectively, of the total oxidase activity, indicating a limited disruption of the mitochondria. The K^+-activated phosphatase had 38% of its activity at low density and 62% at the high density part of the gradient.

Based on these results, the following possibilities were considered: (a) The homogenization was incomplete, the membrane may be naturally attached to intracellular structures. (b) The sarcolemma is bound or adsorbed due to ionic or hydrophobic interactions to other cell organelles, which have higher densities (e.g. with nuclei or myofibrils). (c) The K^+-activated phosphatase is not a unique marker of the sarcolemma. (d) Changes of the density of the membranes occur during preparation.

The first possibility was ruled out when cell homogenates were further treated by ultrasonic irradiation yielding results similar to those obtained following Polythron homogenization only. Furthermore light and electron microscopy showed that both treatments disrupted the cellular integrity.

For the second possibility high concentrations of salt were used to dissolve and extract myosin in order to reduce the interactions of sarcolemma with myosin or actomyosin. Neonatal rat hearts were homogenized rapidly in 0.3 M KCl/0.05 M pyrophosphate, the $10^5 \times g$ pellets were stirred for 30 min at 4°C and fractionated on a plain sucrose gradient. The distribution of the K^+-phosphatase was again, 25% at the low density zone ("top") and 75% at the high density band ("bottom"). Re-extraction of the fractions of the "bottom" with pyrophosphate (42 mM), MgATP (5 mM), or EDTA (5 mM) for 30 min, or with KCl (0.3 M) containing pyrophosphate (0.05 M) for 17 h at 4°C, followed by a plain sucrose gradient, did not alter the banding pattern, considerably. The indication here is that high salt during extraction is not sufficient to produce membranes of lighter density. However, as will be shown later, the composition of the sucrose gradient could lead to a high-density sarcolemma due to changes in the physical properties of the membrane itself.

Enzymatic radio-iodination

As indicated, the use of K^+-activated phosphatase would be misleading if it were not a unique sarcolemmal marker. We decided to use a different marker for probing sarcolemmal components.

The enzymatic radio-iodination of cells under mild, controlled conditions could provide an independent means for exclusive labeling of the external surface of the plasma membranes [28]. This technique has not been applied before to cultured heart cells either in monolayer or suspension. ^{131}I or ^{125}I were added in the presence of glucose oxidase plus glucose to generate H_2O_2 under controlled and continuous conditions [28], with soluble or Sepharose-immobilized lactoperoxidase. Optimal labeling of the cells was obtained with I^- at $2 \cdot 10^{-6}$—$8 \cdot 10^{-6}$ M, incubated for 20—30 min at room temperature. There was no significant difference in the labeling pattern or efficiency whether soluble, or immobilized lactoperoxidase was used. Some difficulties with the beaded enzyme were encountered due to "self iodination" [29] and with the separation of the beads from the cells.

The labeling efficiency ranged from 0.6 to 1.4% of the initial radioactivity,

when 5—50 μCi (i.e. $1 \cdot 10^7$—$10 \cdot 10^7$ cpm) were applied per plate at optimal concentrations of iodine. Each fraction was diluted to reduce the density and subsequently centrifuged or acidified with trichloroacetic acid to reduce possible radio-iodine contamination. Only the radioactivity of the pellets was taken to represent the membranes. The distribution of the labeled membranes was similar to that found in experiments using K^+-activated phosphatase. The radioactive, labeled, particulate fraction, which banded at the zone of high density ("bottom", $d > 1.25$ g/ml) could not be dissociated and "converted" into less dense particles. Attempts to do so included rehomogenization by Polythron or Virtis in an isotonic salt or sucrose medium, and by ultrasonic irradiation [30].

It is possible that structures other than the membrane are labelled. The following results indicate that only the external surface of the sarcolemma was labelled. (1) Sepharose-immobilized and soluble lactoperoxidase gave similar results. (2) Viokase, after iodination, removed 95—97% of the label from the cells.

The protease-treated cells were re-plated and developed into confluent cultures, which beat normally. On several occasions, the cells which were released from their attachment by Viokase and subsequently radio-iodinated enzymatically, while in suspension, took up to 10 times/plate more iodine compared to cells in monolayers. Furthermore, these labeled, intact cells in suspension treated again with Viokase lose about 80% of the radioactivity.

In order to exclude labeling of intracellular components, radioactive cells in monolayers were rinsed carefully and thoroughly, then frozen, thawed, homogenized and centrifuged. The supernatant contained approx. 15% of the radioactivity, yet less than 2% of it was acid insoluble. This indicated that some of the radioactive iodine escaped the rinse or otherwise diffused into the cell but did not cause any significant labeling of the intracellular, soluble proteins (Table III). The intracellular ^{131}I did not iodinate other components effectively due to the impermeability of the auxiliary enzymes. In the absence of one of the auxiliary enzymes, the degree of iodination of the cells was decreased, down to 4 or 10% of the maximum. Addition of serum-containing medium (CMRL-S) or 0.7% bovine serum albumin also reduced the efficiency of labeling by 5 to 10 times.

TABLE III

ENZYMATIC RADIO-IODINATION AND FRACTIONATION OF CULTURED HEART CELLS

Cardiac cells in monolayer cultures were iodinated with ^{131}I in the presence of glucose oxidase, glucose and lactoperoxidase. The radioactive labeled homogenates were centrifuged and the supernatant was treated with 10% trichloroacetic acid.

Fraction	Counts/min in fraction iodinated in presence of			
	Soluble lactoperoxidase		Sepharose-immobilized lactoperoxidase	
	cpm	%	cpm	%
^{131}I cell homogenate	148 000		162 320	
$1.25 \cdot 10^5 \times g$, pellet	115 200	78	122 460	75
$1.25 \cdot 10^5 \times g$, supernatant	21 655	15	24 000	15
Trichloroacetic acid insoluble	2 685	1.8	2 110	1.3

We have analyzed the homogenates fractionated on a sucrose density gradient for lipids following extraction by chloroform and methanol [22,23]. The radioactivity in the upper, aqueous methanolic phase represents non-lipid components whereas that of the lower, chloroform phase represents lipids. With the correction for the quenching of radiation by chloroform (a factor of 1.6), the ratio of radioactivity in the upper to lower phases ranged from 8.5 to 17. These values were obtained with the unfractionated homogenates as well as with the fractions collected from the sucrose density gradients. We therefore feel that under these circumstances the efficiency of labeling the lipids of the sarcolemma of cultured heart cells in monolayers is quite limited (cf. refs. 31 and 32).

Since the cultured heart cells are a primary culture, a mixed cell population is expected. Muscle-enriched cultures were prepared by differential attachment and grown to confluency. These enriched cultures contained more beating cells and a higher content of myosin than the plates with fewer muscle cells (Table IVA). Muscle-enriched cultures were labeled with iodine two times higher, and non-muscle cultures two times lower, than the mixed cultures from which they were derived (Table IVB).

To describe the pattern of distribution of the marker between low and high density regions, a distribution index (D.I.) was employed which is defined as the ratio of the measured quantity in the low density fraction divided by the quantity in the high density fraction, i.e. D.I. = Low density/high density =

TABLE IV

RADIO-IODINATION OF MUSCLE AND NON-MUSCLE CELLS

Neonatal rat heart cells were plated as such (mixed cells) or differentially separated by preplating for 45 or 90 min. The non-adhering cells (muscle) were decanted and plated separately; the adhering cells (non-muscle) were also grown. Cell counting was done following Viokase proteolysis in EDTA using a "particle counter". The total muscle cell content was determined by: (a) fluorescent antimyosin antibodies [13] and (b) myosin Ca^{2+}-ATPase following extraction with high concentration of KCl [21]. Units are expressed as nmol P_i produced from ATP per min. Protein was determined as outlined in Methods. (A) A representative experiment of a partial separation of muscle from non-muscle cells. (B) ^{125}I labeling of muscle cell- and non-muscle cell- enriched plates. The numbers in parentheses represent number of experiments.

A Cell type	Time of preplating	Cells $\times 10^6$/plate	Total muscle cells		Protein (mg/plate)	Myosin (units/plate	ATPase (units/mg protein)
			$\times 10^6$/plate	%			
Mixed	0 min	2.885 ± 0.126 (3)	0.808	28	0.97	76	78
Non-muscle	45 min	2.046 ± 0.141(3)	0.286	14	0.375	13	35
Non-muscle	90 min	2.091 ± 0.115 (2)	0.251	12	0.364	16	44
Muscle	45 min	1.623 ± 0.108 (3)	0.698	43	0.7	88	126
Muscle	90 min	1.015 ± 0.032 (3)	0.477	43	0.61	70	115

B Cell type	Counts/min		
	Experiment 1	Experiment 2	Experiment 3
Mixed	450 000 (1) 100%	297 000 (2) 100%	713 000 (5) 100%
Non-muscle	—	257 000 (2) 87%	342 000 (5) 48%
Muscle	1 350 000 (1) 300%	646 000 (2) 217%	992 000 (5) 139%

top/bottom. The distribution of markers from heart cell homgenates and from isolated sarcolemma, on a gradient, using fresh or frozen material was determined. The results for the unfrozen preparations fell into two groups, (a) which showed a D.I. smaller than 1 and (b) which showed a D.I. value greater than 1. They are represented as two separate groups (Table V) and demonstrate that there is almost an equal probability of having D.I. values greater or smaller than 1, with all three measured parameters. The frozen preparations, however, consistently showed D.I. values lower than 1.

Effects of high concentrations of salt

On a plain sucrose gradient, irreproducible distribution of ^{125}I-labeled sarcolemma was observed whether the cells were homogenized in isotonic ionic or non-ionic medium or extracted for extended periods of time with high concentrations of salt. These results agree with the data for K^+-phosphatase. However, when these homogenates were extracted overnight with the high concentrations of salt (e.g. 0.3 M KCl containing 0.05 M pyrophosphate) and subsequently fractionated on a sucrose gradient containing the same concentrations of high salt, more frequently the ^{125}I-labeled sarcolemma banded at the top of the gradient (e.g. $d < 1.16$ g/ml). Under these circumstances the D.I. values with fresh, unfrozen homogenates were higher and freezing the membranes again yielded lower D.I. values (Table VI). However, even in these preparations salt treatment increased the D.I. The high salt concentration did not affect the activity of the K^+-activated phosphatase (data not shown). Salt solution present from the beginning with fresh tissue, in which the density of the membrane has not changed, preserves the original density but after freezing has changed the density, the effect of salt treatment is limited.

Radio-iodination of other cells and isolated sarcolemma; interactions with heart cell homogenates

It was thought important to utilize the effect of freezing and thawing and

TABLE V

DISTRIBUTION INDEX OF HEART SARCOLEMMA

Heart cell homogenates were fractionated on a continuous density gradient of sucrose. The homogenization was done by Polythron, Dounce or ultrasonic irradiation in the presence of H_2O, Tyrode-G, ATP, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic (EGTA) or high concentrations of KCl, and the fractionation was done with either fresh or frozen and thawed preparations. The amount of radioactivity (cpm of ^{125}I), K^+-activated phosphatase and protein in the fractions of the gradient at the "top" and "bottom" (densities < 1.16 and > 1.21 g/ml, respectively), are expressed in units of Distribution Index. This unit (D.I.) is defined as the ratio of the measured quantity in the top and bottom of the gradient. The number of experiments is given in parentheses.

Homogenate	Distribution index (D.I.)			
	^{125}I radioactivity		K^+-activated phosphatase	Protein
	(a)	(b)		
Fresh	28 (7)	0.33 (7)	0.88 (1)	3.1 (1)
Frozen and thawed	0.43 (4)		0.83 (1)	0.74 (1)

TABLE VI

EXTRACTION OF HEART CELL HOMOGENATES WITH KCl AND PYROPHOSPHATE

Experiments A and B: Radio-iodinated heart cell homogenates kept frozen, were thawed and aliquots were applied to a plain sucrose gradient or alternatively to a gradient containing a solution of 0.3 M KCl, 0.1 M Tris, 0.05 M pyrophosphate and 1 mM EDTA, pH 7.4 (sucrose + salt), following an overnight stirring in the same solution. Experiment C: Sarcolemma was isolated from cultured cell homogenate by density gradient centrifugation (the "top" fractions). It was subsequently radio-iodinated in suspension and aliquots were either immediately recentrifuged on a fresh, plain sucrose gradient or in a salt-containing gradient following an overnight stirring in the salt solution described above.

Expt.	Treatment	Distribution index of cpm in the following gradient	
		Sucrose	Sucrose + salt
A	Frozen and thawed	0.04	0.50
B	Frozen and thawed	0.07	0.134
C	Fresh	0.12	6.00

of the salt concentration on the isolated sarcolemma in order to see whether the sarcolemma was structurally attached to internal dense fractions of the cell or whether the sarcolemma was joined to the denser fractions subsequent to the disruption of the cell. Non-labeled membranes obtained from the top fraction of sucrose gradient of heart cell homogenates and from hemolyzed erythrocytes were enzymatically iodinated. These labeled membranes were centrifuged with or without freezing, with or without heart cell homogenates, and with or without high salt treatment as indicated in Table VII. Erythrocyte membranes behaved "normally", with or without high salt treatment (Table VIIA). However, freezing of the erythrocytes increased their density and the presence of heart cell homogenates had no effect on their density distribution (Table VIIB). Heart cell membranes when frozen also became denser and the presence or absence of heart cell homogenates had little effect (Table VIIC, D3). However, fresh heart cell membranes in low salt had a D.I. below 1 and the presence of heart cell homogenates slightly increased the D.I. (Table VII, D1). If, however, fresh membranes are prepared and separated with high salt the D.I. is high (Table VII, D2) and is not affected by the presence of heart cell homogenates. These results lend weight to the possibility that the presence of cellular components may cause the sarcolemma to adhere to higher density fragments, but that the presence of salt prevents this adherence. Freezing of the heart cell membrane causes a change in the density which seems to be prevented by the presence of high salt. Freezing of the ^{125}I-labeled membranes alone can cause a change in their density. This indicates that the distribution of the sarcolemma in preparations of heart cells may have a low D.I. value not only because of their attachment to denser particles but because of changes in their properties.

Cultured liver cells in monolayer were enzymatically iodinated. Their frozen homogenates when thawed and fractionated, behaved like the cardiac cells exhibiting a D.I. of 0.3. On the other hand, erythrocytes were iodinated in suspension, and their frozen, thawed and fractionated homogenates gave D.I. values greater than 1.

TABLE VII

INTERACTIONS OF IODINATED SARCOLEMMA OR ERYTHROCYTE MEMBRANES WITH CARDIAC CELL HOMOGENATES

Membranes obtained either from heart cell homogenates and isolated from the "top" fractions of the sucrose gradient, or from hemolyzed erythrocytes, were enzymatically radio-iodinated. The labeled membranes were fractionated immediately or following freezing and thawing, on a sucrose gradient. Expt. A: A suspension of fresh ^{125}I-labeled erythrocyte membranes was homogenized with cultured heart cells, stirred overnight with salt solution (cf. Table VI) and fractionated on a sucrose gradient in the absence or presence of the salt solution. Expts. B and C: A suspension of frozen and thawed ^{125}I-labeled membranes was added to the cultured cells in petri dishes either before, during or after the scraping and homogenization of the cells. They were subsequently fractionated on a plain sucrose gradient. Expt. D: Either freshly isolated or frozen and thawed ^{125}I-labeled sarcolemma was homogenized with or without cultured cells and fractionated on a sucrose gradient. Comments: (1) Fractionation of salt-treated homogenates was done in a gradient containing salt. (2) The number of experiments is given in parentheses.

Expt.	Source of ^{125}I-labeled membranes	Treatment of membranes		Distribution index of ^{125}I-labeled membranes	
		Freezing	Salt	Without heart homogenates	With heart homogenates
A	Erythrocytes	—	±	>>1	>>1 (2)
B	Erythrocytes	+	—	0.2	0.13 (2)
C	Heart cells	+	—	0.2	0.34 (6)
D 1	Heart cells	—	—	0.12	0.81 (2)
2	Heart cells	—	+	6.00	5.60 (2)
3	Heart cells	+	—	0.22	0.16 (2)
4	Heart cells	+	+	1.00	7.09 (2)

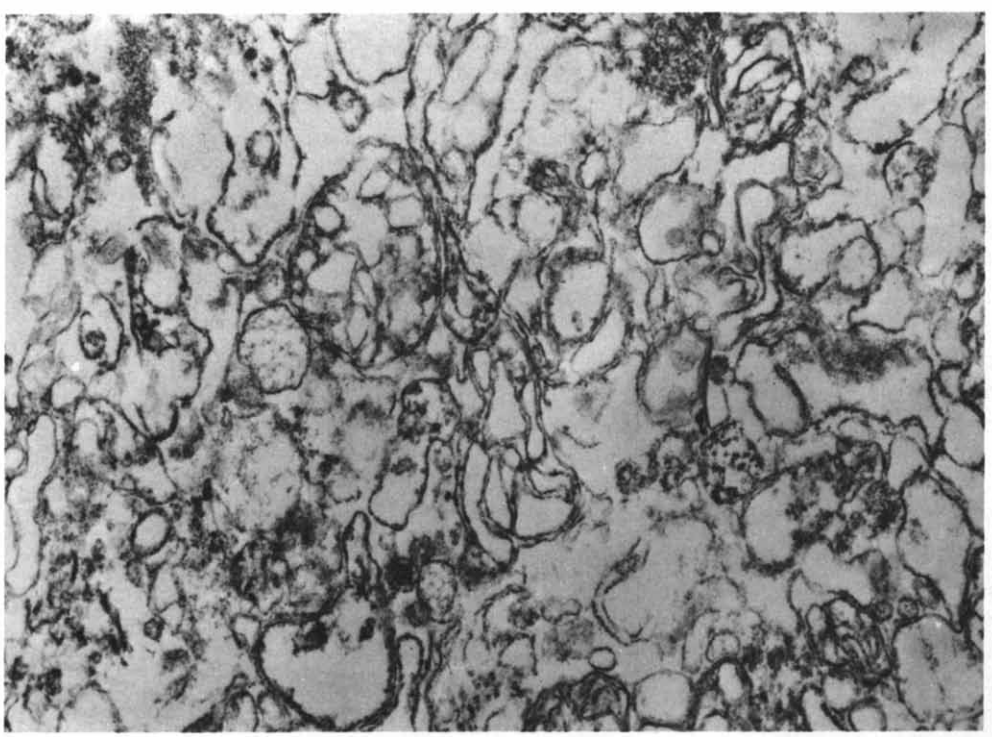

Fig. 1. Electron micrograph of the "top" fraction of the density gradient, following overnight stirring with 0.3 M KCl in the presence of 0.05 M pyrophosphate. Total magnification × 32 000.

Electron microscopy

Electron microscopic examination shows that the fractions collected from the dense part of the sucrose gradient (bottom) contained a mixture of myofibrils, fragmented nuclei, a few mitochondria, and numerous vesicles displaying a wide range of sizes and some of them looked like "rough" microsomes. The fractions collected from the low density portions of the gradient (top), were enriched with sarcolemma vesicles, especially after extraction with salt (Fig. 1).

Discussion

Studies on the molecular architecture and biochemical composition of the isolated cardiac sarcolemma await the preparation of pure sarcolemma in high enough yields [33]. For this, one needs unique markers and procedures which yield intact, stable preparations. The presence of specific enzymes has been frequently used to identify cellular membranes. It has, however, to be established that an enzyme is preferentially associated with the particular subcellular organelle. Wollenberger and Schulze, using cytochemical techniques of lead precipitation, showed that the enzyme ($Na^+ + K^+$)-ATPase, which was ouabain sensitive, can be identified only at the cardiac sarcolemma [34]. Another enzyme considered to be part of the sarcolemma is the adenylate cyclase, which has been shown to be located along the entire course of the cardiac plasma membrane, at the transverse tubules and at the tight junction region of the intercalated discs [34].

In other studies, in which cardiac tissue was homogenized and fractionated, the results are not always as clear-cut as in the cytochemical studies. Drummond et al. [4] using low speed centrifugation of cardiac homogenates and a step of salt extraction followed by a non-continuous sucrose gradient centrifugation, showed the adenylate cyclase to be associated with a subcellular fraction with density higher than 1.266 g/ml. These particles retained 13% of the ($Na^+ + K^+$)-ATPase and 5% of cytochrome *c* oxidase. In a similar study, Moffett et al. [6] used the single step, continuous density gradient centrifugation method of Kidwai et al. [5]. By this method it was claimed, the plasma membranes of the heart homogenates banded at a density region of $d < 1.15$ g/ml which contained most of the ($Na^+ + K^+$)-ATPase and the K^+-activated phosphatase [5]. However, the recovered adenylate cyclase in the later study [6], was found primarily in the heavier fraction which banded at a density of $d > 1.23$ g/ml.

Our studies indicate that these contradictory results may also arise because of the adherence of the plasma membrane to denser cellular fractions. In the present experiments we have attempted to re-evaluate the controversial data by using independent markers of the sarcolemma in addition to the enzymatic markers. We have employed the K^+-activated phosphatase which is inhibited by ouabain, as a marker, for the reasons that it is easily assayed and has been shown to be associated with plasma membranes in several tissues [35]. From our own studies on erythrocyte membranes [36] as well as others, the plasma membranes were described as having a density around 1.15 g/ml. We have added a third, independent probe by labeling the sarcolemma of the intact cardiac cells

in culture with radioactive iodine, under mild specific conditions [28]. Our data excludes the labeling of the intracellular, soluble proteins which justifies also the assumption that no other intracellular membranes of the intact cells became labeled with radioactive iodine. We have also shown that this process is mild enough not to affect the viability of the cells. The observation that the cardiac muscle cells can be radioactivity labeled to a greater extent than the non-muscle cells, is worth emphasizing. This may result from the possibility that muscle cells have a larger surface area, a higher content of phenolic rèsidues or a higher lipid content. Our data tend to rule out the role of heavy labeling of the lipid fraction of muscle cells since they had a low level of lipid labeling.

We have emphasized in the present study that conflicting results similar to those published by Kidwai and coworkers [5,6] are easily obtained. In our studies on isolated membranes the variation in density of the sarcolemma, identified by enzymes or radioiodine, caused by freezing and thawing, high salt extraction or by the presence of cell homogenates indicate that the sarcolemma exists in a metastable state. This instability may very well be the cause of variable results. Sarcolemma is best isolated working rapidly with the fresh material in the presence of high salt. Using such a procedure the sarcolemma as identified by enzyme markers and iodination is in the expected position on the density gradient of either sucrose or Ficoll. Electron micrographs support this pattern. These lighter fractions contained mostly membrane vesicles whose sizes varied as a function of the treatment, whereas the denser fractions contained fragments of nuclei, myofibrils as well as occasional membrane vesicles.

Early workers introduced a high salt extraction step to remove so called "sticky" contractile proteins. However, this approach was criticized on the ground that the salts inactivate most membrane enzymes unless used in the presence of isotonic sucrose [2,37]. We have confirmed the earlier findings that prolonged extraction with relatively high concentrations of KCl, for instance, which is employed for the extraction of myosin, resulted in considerably higher yields of "scarcolemma" in the proper position on the gradient. However, this occurred more frequently with freshly prepared homogenates whereas frozen preparations, for example, did not tend to alter their already high density even after the salt extraction. Furthermore, this behavior was not peculiar to heart cells but was also observed with erythrocyte membranes or cultured liver cells. We therefore suggest that cardiac sarcolemma and possibly plasma membranes from other sources tend to alter some of their physical properties (e.g. density) during preparation and/or subsequent handling. It should be stressed again, how important it is to define the exact conditions of preparation and handling of the membranes or other sub-cellular components for any biochemical, physiological, pharmacological or morphological problems they might serve and especially, whether the membrane fraction meets the criteria of purity, morphology, recovery and represents adequately that particular plasma membrane. Our results also indicate that the adherence of the sarcolemma to the denser fraction is probably not a result of a normally present structural continuity with the cellular organelles but most likely a result both of the properties of myosin and of the membranes which could result in passive adherence after homogenization.

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