ISOLATION OF PLASMA MEMBRANE FROM CARDIAC MUSCLE

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Received August 30, 1971

SUMMARY

A method of preparing relatively pure plasma membrane from cardiac muscle is described. This and other subcellular fractions can be obtained from a single density gradient step. 5'-nucleotidase, $Na^+ + K^+$-ATPase and K^+-activated phosphatase were found to be concentrated in the plasma membrane fraction. The ratio of $Na^+ + K^+ + Mg^{++}$ ATPase to that of Mg^{++}ATPase was increased to 2.0 by treatment with deoxycholate. Electronmicroscopic studies revealed the presence of vesicles in plasma membrane fraction. Sarcoplasmic reticulum and mitochondrial fractions were also relatively pure when examined electronmicroscopically.

High purity, good yield, fast processing and absence of any salts at high concentrations make this method very useful for pharmacological and biochemical studies.

Previous work from this laboratory (1) has demonstrated that isolation of plasma membrane does not necessarily require extraction of tissue homogenate with high concentrations of salts as reported by many in the case of skeletal muscle (2). Attempts have been made to subfractionate the microsomal fraction of heart into sarcoplasmic reticulum and plasma membrane (3,4) but the yield of plasma membrane and cross contamination are serious disadvantages. In the present communication we have applied a similar method to that for isolation of plasma membrane of rat uterus to rat heart. Using the same technique in heart tissue we found that most of the contractile proteins under our conditions settle down in the bottom of the density gradient tube and do not interfere with the plasma membrane preparation. Despite the importance of heart muscle subcellular fractions for studies on Ca^{++} uptake, and $Na^+ + K^+$-ATPase no satisfactory method of isolation of plasma membrane

and other subcellular fractions was found in the literature which can be applied to heart muscle. The present method has the advantage of being fast, gentle, providing a high yield of plasma membrane and recovery of all the subcellular components in a single step density gradient centrifugation.

MATERIALS AND METHODS

Female Wistar rats of 220 gm-240 gm wt. range were killed by a blow on the head and hearts were removed immediately and placed in cold (0-4°C) 0.25 M sucrose prepared in glass distilled water. They were then washed with more sucrose to remove blood and 5 g of heart tissue was homogenized in Polytron PT-20 (Brinkmann Instruments Inc., N.Y.) for 15 sec. at half maximum speed. A 20% homogenate was prepared and filtered through a wire cloth mesh 40 (Small Parts Inc., 6901 N.E. Third Ave., Miami, Florida.) under mild suction. The filtrate was centrifuged at 100,000 xg for 30 min in Spinco Model L_2 Centrifuge. The sediment was suspended in 0.25 M sucrose in a small volume and loaded on top of the gradient and centrifuged for 90 min at 111,688 xg as described by Kidwai et al[1]. The top band (F_1) was plasma membrane, the hazy area below the band was mainly sarcoplasmic reticulum (F_2) and a thick sharp band in the middle of the tube was mitochondria (F_3) as judged by electronmicroscopy. Under the mitochondrial fractions there were cell debris and nuclei (F_4). The contractile proteins were at the bottom of the tube (F_5) gelatinous in nature and soluble in high concentrations of KCl. Occasionally a red band of blood cells was observed just above the contractile proteins. Various bands were removed by Pasteur pipettes diluted to a sucrose concentration of 0.25 M and recentrifuged at 100,000 xg for 30 min. The sediment resuspended in sucrose or buffer as required or the pellets were fixed with buffered glutaraldehyde (pH 7.1) for electronmicroscopy[1].

$Na^+ + K^+$-activated ATPase was determined in a reaction mixture of 1 ml containing 50 mM Histidine-HCl buffer pH 7.4, 3 mM MgCl, 3 mMATP (Mg salt) 100 mM NaCl, 10 mM KCl. The reaction was started by adding the ATP and the mixture was incubated at 37°C for 15 min. Reaction was stopped by adding 1 ml of 10% TCA, centrifuged and inorganic phosphate liberated was determined by Fiske and SubbaRow[5].

The ratio between the ATPase activity due to $Mg^{++} + Na^+ + K^+$ and that due to Mg^{++} alone was increased by treating the F_1 pellet for 20 min with a solution containing 0.25 M sucrose, 0.15% sodium deoxycholate, 0.2% Histidine, 5 mM Na EDTA pH adjusted to 6.8 with HCl. The treated material was centrifuged for 30 min at 100,000 xg, resuspended in 0.25 M sucrose and centrifuged as before. The sediment was suspended in the same molarity of sucrose and frozen until used.

Cytochrome c oxidase, 5'-nucleotidase and K^+-activated phosphatase were determined as described earlier[1].

Protein was determined by Lowry's Method[6].

Preparation of the density gradient was similar to that reported by Kidwai et al.[1].

RESULTS AND DISCUSSION

This method of preparation of subcellular fractions from heart is simple and fast and is a modification of an earlier method[1]. Filtration of the homogenate through wire cloth mesh 40 helps to remove most of the connective tissue. The equilibration time on the density gradient was reduced in the case of heart muscle compared to smooth muscle since mitochondria moved down the density gradient faster. No mitochondria could be seen in the plasma membrane fraction under electronmicroscopy after a centrifugation on the density gradient for 90 minutes. Plasma membrane obtained at the top of the gradient was vesicular when examined in the electronmicroscope and a triple layer structure was evident in most of the preparations. 1.2% of the total protein was recovered in the plasma membrane fraction. ATPase activity stimulated by $Na^+ + K^+$ and

Table 1. Average specific activities of $Na^+ + K^+$-activated ATPase of plasma membrane fraction

μmoles Pi released per mg protein/hour at 37°C.

Additions	Untreated plasma membrane fraction		Deoxycholate treated plasma membrane fraction	
Mg^{++}	79	(5)	13	(3)
$Mg^{++} + Na^+ + K^+$	94	(5)	26	(3)
$Mg^{++} + Na^+ + K^+$ + ouabain 10^{-3}M	76	(5)	13	(3)

Numbers in parentheses are number of determinations each from a separate preparation.

inhibited by ouabain was present only in this fraction. Other fractions did not show any significant Na^+, K^+ stimulation of ATPase even after deoxycholate treatment. This could be due to different composition of the subcellular fractions. K^+-activated phosphatase sensitive to ouabain and 5'-nucleotidase activities were also concentrated in this fraction as shown in Table 2. ATPase activity due to Mg^{++} alone was 79.0 µmoles Pi released per mg protein per hour. In the presence of $Na^+ + K^+$ the activity was 94.0; adding 10^{-3}M ouabain decreased the activity to almost the basal level (Table 1). Treatment of this fraction with deoxycholate increased the ratio between the Mg^{++}, Na^+, K^+-activated enzyme to that of Mg^{++} activated enzyme to approximately 2.0.

K^+-activated and ouabain sensitive phosphatase activity was 0.81 in plasma membrane and 0.24, 0.20 in F_2 and F_3 respectively. This enzyme is considered to be part of $Na^+ + K^+$-stimulated ATPase[7] and therefore used as a marker enzyme for plasma membrane fraction. Enrichment of this enzyme activity in fraction F_1 supports the evidence of the presence of plasma membrane in fraction F_1 (Table 2).

5'-nucleotidase is considered to be a plasma membrane marker enzyme[8], however, its presence in other fractions cannot be excluded[9]. Our observations are consistant with the findings of others[8] that 5'-nucleotidase is concentrated in the plasma membrane. Our plasma membrane preparation had five times higher specific activity of 5'-nucleotidase as compared to that of homogenate, (Table 2).

Cytochrome c oxidase activity was mainly confined to F_3 which supports our electronmicroscopic observation of this fraction as being mitochondrial in nature, however some cytochrome c oxidase activity was present in F_1 and F_2 which could be due to the mitochondrial fragments present in those fractions. It was not possible to determine the activity in the homogenate due to the turbidity interference in the spectrophotometric method used (Table 2).

In the absence of any accepted sarcoplasmic reticulum markers we were

Table 2. Average specific activities of plasma membrane and mitochondrial marker enzymes in the subcellular fractions of cardiac muscle

Fractions	5'-nucleotidase	K^+-activated p-nitrophenylphosphatase	cytochrome c oxidase ΔE 550/min/mg protein
Homog.	3.6 (2)	0.35 (3)	——
F_1	17.0 (3)	0.81 (4)	21 (2)
F_2	5.1 (2)	0.24 (3)	21 (2)
F_3	1.1 (3)	0.20 (2)	166 (2)

Numbers in parentheses are number of determinations each from a separate preparation.

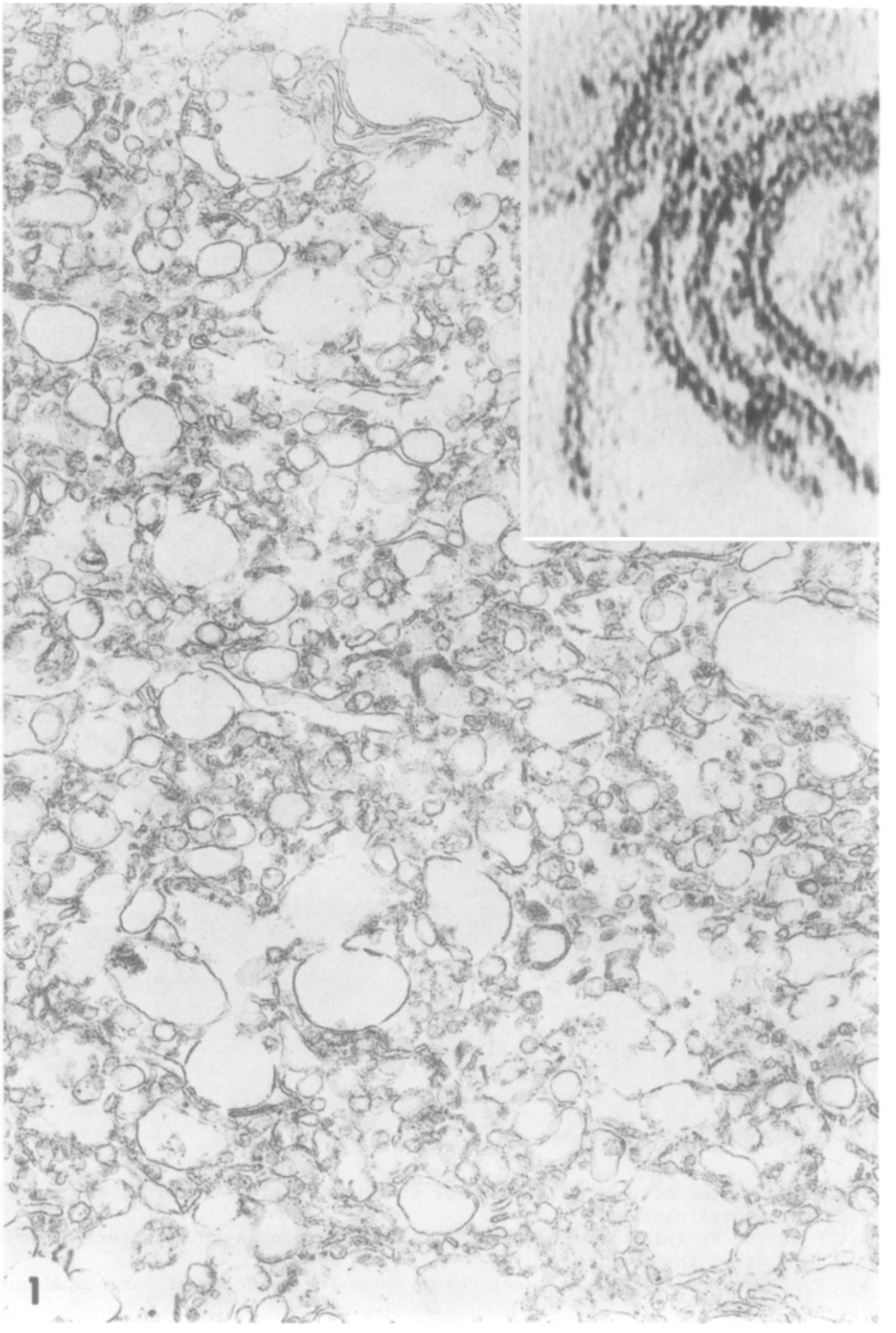

Fig. 1. The electron microscopic picture of a thin section prepared from the pellet of F_1. Plasma Membrane in the form of vesicles. Magnification 40 x 10^3. Inset shows the triple layered structure, 350 x 10^3.

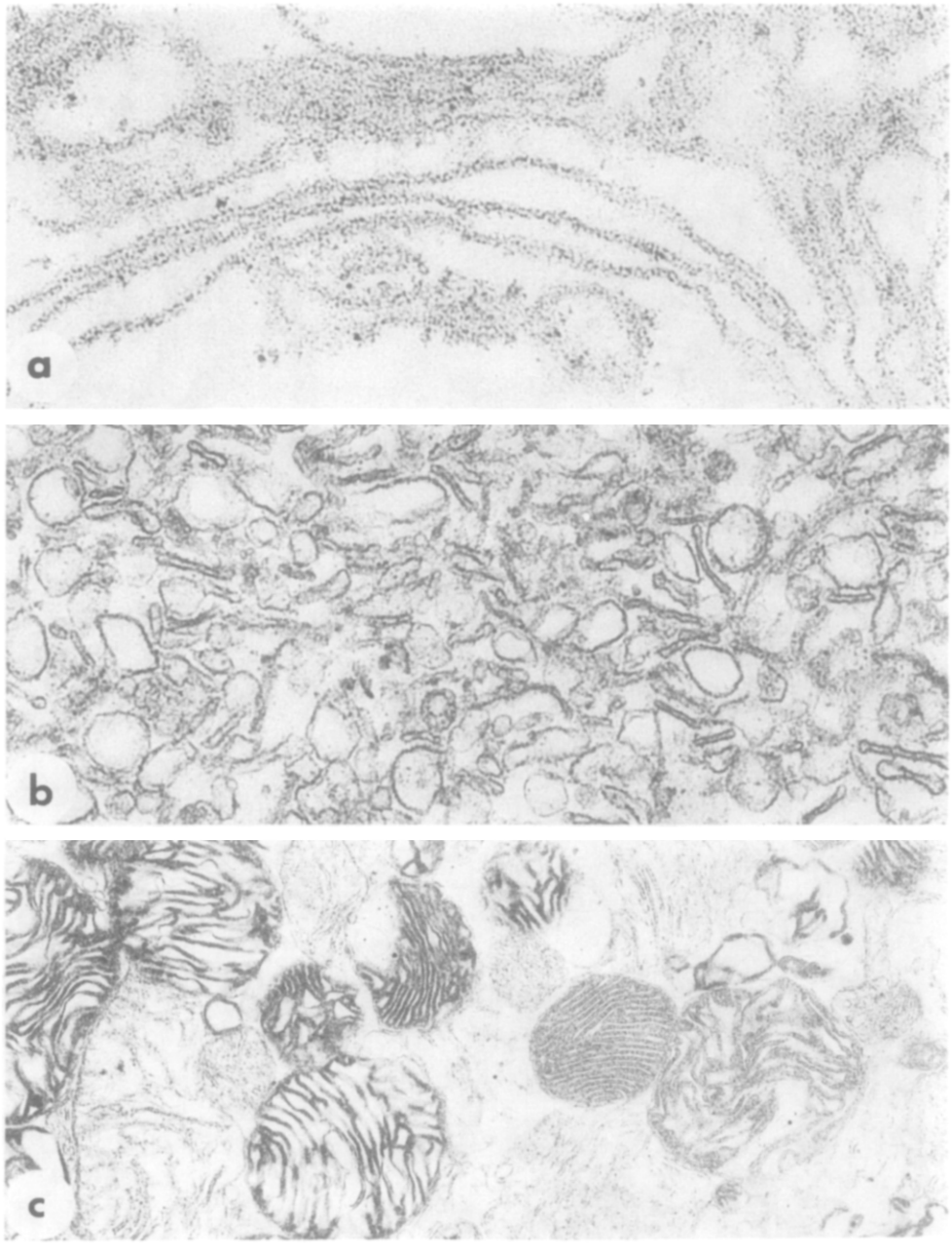

Fig. 2. Photoelectron micrographs of various fractions isolated after density gradient centrifugation.
a. Fraction F_1 (plasma membrane) 200 x 10^3. Triple layer structure is evident in most of the membranes.
b. Fraction F_2 (sarcoplasmic reticulum) 60 x 10^3 vesicles of various shapes and sizes.
c. Fraction F_3 (mitochondria) 20 x 10^3

unable to determine the extent of cross contamination of plasma membrane by this fraction.

Electron microscopic observations revealed the distribution of other subcellular components in the density gradient tube from the top. The plasma membrane was at the top (F_1) in the form of vesicles with a characteristic triple layer structure Fig. 1 and Fig. 2a. The next hazy wide band was sarcoplasmic reticulum (F_2)(Fig. 2b) followed by the mitochondrial band (F_3) Fig. 2c and nuclear fraction (F_4)(picture not included). The bottom of the tube had most of the contractile proteins which were soluble in high concentrations of KCl and precipitated on dialysis. Occasionally blood was present which formed a red band just above the contractile proteins at the density gradient step.

In conclusion, this method of plasma membrane preparation from rat heart muscle is fast; that is the membrane can be prepared in less than three hours, does not require exhaustive washings with high salt concentrations, and provides a good yield of plasma membrane and other subcellular fractions in a single step using the density gradient. This preparation was useful in studying membrane bound enzymes e.g. $Na^+ + K^+$-ATPase which can be further purified from this fraction by the use of deoxycholate alone. Salt extraction as reported by Tai Akera et al.[10] was not necessary. Plasma membrane and other subcellular components isolated can be used for drug-receptor interaction, transport functions, and a variety of biochemical and pharmacological studies.

ACKNOWLEDGMENTS

This work was supported from the grants by Alberta Heart Foundation and Medical Research Council of Canada.

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