STRUCTURE - FUNCTION RELATIONSHIP IN THE ISOLATED RAT HEART SARCOLEMMA: A CYTOCHEMICAL AND BIOCHEMICAL STUDY.

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by

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ABSTRACT

Appreciation of the interrelationship between structure and function in biological membranes is fundamental to the understanding of cellular processes in both normal and pathological states. In the present investigation this relationship was studied in two isolated membrane preparations, from rat hearts, believed to be derived from the myocardial sarcolemma. The first part of this project focuses on a comparative study of the two membrane preparations and is followed by an in depth investigation of one of them.

Both preparations displayed properties which are characteristic of the sarcolemma, however, distinct differences in morphology, cytochemical staining patterns and enzyme activities, calcium binding activity and sialic acid content were noted.

Method I membranes, obtained by the procedure of McNamara et al (1974) as modified by Dhalla et al (1977), were comprised of vesicles of variable shape and size with a visible cell surface layer external to the unit membrane. This layer was stained when either colloidal iron (CI), lanthanum or ferritin was applied to the isolated preparation and also when lanthanum or ferritin was perfused through the heart prior to membrane isolation.

Method II membranes, prepared essentially according to the sucrose density gradient procedure of Kidwai et al (1971), consisted of small vesicles without any readily apparent cell surface material. These vesicles did not bind any of the three cationic probes utilized to any significant degree. The results

of the cytochemical study strongly suggested that Method II membranes did not possess the glycoprotein rich surface coat. This contention was further supported by the failure to find any appreciable amount of sialic acid in this preparation. The Method I sarcolemmal preparation, in contrast, contained a significant amount (40.4 ± 3.14 nmoles/mg protein) of sialic acid. Furthermore, 60% of the total membrane sialic acid was hydrolyzed by neuraminidase in this preparation, indicating that most sarcolemmal vesicles were oriented in the right side out conformation.

Enzyme activities of the two preparations were qualitatively similar, however, only Method I membranes displayed any significant sensitivity of the Na^+-K^+ATPase to ouabain. This finding suggested that Method II vesicles might be oriented in the inside out conformation. In support of this suggestion, calcium binding by Method II membranes was not inhibited in the presence of high concentrations of Mg^{2+}. In fact, this preparation displayed enhanced calcium binding activity in the presence of Mg^{2+} and ATP. This type of activity has been reported by other workers who believe it may represent the sarcolemmal calcium pump which translocates this cation to the extracellular milieu. In addition, membranes displaying this kind of calcium binding activity are believed to be oriented inside out. Method I sarcolemmal membranes were significantly inhibited in the presence of Mg^{2+} and ATP. This type of inhibition in the presence of high concentrations of Mg^{2+}, is characteristic of the

extracellular calcium binding pool, which is believed to be necessary for excitation -contraction coupling in cardiac cells.

Since in this part of the investigation Method I membranes displayed characteristics more like the sarcolemmal membrane in vivo, further investigations into the structure and function relationship of this membrane preparation were conducted.

Studies on Method I membranes included colloidal iron staining, enzymatic activities and calcium binding activity both before and after treatment with either trypsin, phospholipase C or neuraminidase. This investigation was undertaken in order to: a) assess the contribution of proteins, phospholipids and sialic acid to the surface negative charge and b) to study the effect of removing or modifying these membrane components on sarcolemmal enzyme and calcium binding activities.

Colloidal iron staining was reduced by all three enzyme treatments, but no one treatment was effective in completely preventing the subsequent binding of the stain to the sarcolemmal membrane. Membrane enzyme activities responded in a variable fashion to these treatments. Na^{+}-K^{+}ATPase activity was reduced by all three interventions with the order of potency in inhibiting this activity being trypsin > phospholipase C > neuraminidase. Trypsin treatment resulted in an increase in the activities of both Mg^{2+} and Ca^{2+} ATPases, while phospholipase C caused a slight depression in the activities of these two enzymes. Neuraminidase caused a uniform decrease (30-40%) in the activities of all three enzymes studied and this observation may indicate involvement of

the surface charge in the regulation of membrane bound enzymes.

Membrane calcium binding was reduced by trypsin and neuraminidase to both high and low affinity calcium binding sites while phospholipase C caused a siginificant depression in calcium binding to only the low affinity site. The results of the present investigation regarding both the cytochemical staining and calcium binding characteristics provide strong evidence that a mosaic of biomolecules contribute to both the surface negative charge and to calcium binding sites.

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INTRODUCTION

Elucidation of the structure-function relationship of the myocardial cell membrane or sarcolemma is a necessary prerequisite to a more complete understanding of both normal and pathological processes in the mammalian heart. Myocardial sarcolemma has in common with the membranes of other cells a characteristic structure consisting of a lipid bilayer containing both extrinsic and intrinsic protein moieties with the distinction being based on the degree of association of the protein molecules with the hydrophobic lipid core (Singer and Nicolson, 1972). In addition, an amorphous layer external to the lipid bilayer, consisting of glycoproteins and mucopolysaccarides forms an integral part of the sarcolemmal membrane complex (McNutt, 1975; Langer, 1978).

Although studies on the isolated membrane preparation have added to the understanding of sarcolemmal functions, a great deal of contradictory evidence as regards both the qualitative and quantitative properties of heart plasma membranes has appeared in the literature. Such differences may be related to species variation, contamination of the isolated fraction with other cellular components, alteration of membrane properties by the isolation procedure or differing orientation of membrane vesicles resulting in the manifestation of properties associated with the cytoplasmic or extracytoplasmic sides of the sarcolemma. Functional assymetry of biological membranes suggests activities associated with each side of the membrane would be expected to

differ. These factors or a combination of any or all may account for conflicting reports.

In view of these factors and their probable influence on the properties of isolated membrane fractions, the first part of the present study was designed to examine both the structural and functional characteristics of heart cell membrane preparations isolated from rat myocardium by two different procedures, to determine if structural alterations underlie variations in function. Calcium binding, enzyme activities and sialic acid content, both neuraminidasè sensitive and total, were examined in both preparations. Morphology and the staining characteristics of both sarcolemmal preparations in the presence of colloidal iron, lanthanum and ferritin were also studied. The rationale behind this approach was that by utilizing the same species and by choosing two isolation procedures which have been shown to yield plasma membrane preparations substantially free of other cellular contaminants we would be able to assess the contribution of isolation procedure to both the structural and functional differences of these two preparations.

Furthermore, many studies have appeared on the relationship between sarcolemmal structure and function (Langer, 1978;Singal et al, 1980). Enzyme proteins such as the Na^+-K^+ATPase and adenylate cyclase, which function to maintain membrane excitability and the metabolic, chronotropic and inotropic responses to catecholamines respectively, have been suggested to be localized in the plasma membrane (Dhalla et al, 1977; 1978).

Although changes in membrane structure could account for functional abnormalties observed in some pathological states, experimental evidence correlating structure-function relationships in the sarcolemma seems to be lacking.

The presence of a fixed negative charge at the cell surface (Elul, 1967) is believed to be responsible for the binding of cations, notably calcium, in the heart (Dhalla et al, 1977;Langer et al, 1976;Langer, 1978). The ability of other cations, such as lanthanum, to uncouple excitation from contraction, presumably by replacing calcium at the cell surface (Sanborn and Langer, 1970;Shine et al, 1971), indicates calcium is bound to negatively charged membrane sites. Sialic acid, a nine carbon amino sugar, has been shown to bind calcium in a preferential fashion (Jaques et al, 1977) and is believed to regulate the supply of contractile dependent calcium (Howse et al, 1970;Langer, 1978). It is thus imperative to understand the distribution and source of the fixed negative charge at the sarcolemma if we hope to understand calcium metabolism in the heart.

The second aspect of the present study was undertaken to investigate the distribution of negatively charged sites on the isolated rat heart sarcolemma by electron microscopic cytochemical procedures. Furthermore, membranes were treated with enzymes such as neuraminidase, trypsin and phospholipase C to assess the contribution of sialic acids, proteins and/or glycoproteins and phospholipids respectively to these negatively charged sites. The effect on membrane calcium binding and

enzymatic activities related to changes in the density and distribution of negative charge following these treatments was investigated.

REVIEW OF THE LITERATURE

The cells of the mammalian myocardium are invested by the sarcolemma which is composed of the 7.5 nM lipid bilayer described by Singer and Nicolson (1972) and an adherent surface coat termed the "glycocalyx" (Bennett, 1963; McNutt, 1975; Langer, 1978). The sarcolemma functions as a semi-permeable barrier between the extracellular and intracellular compartments (McNutt, 1975; Dhalla et al, 1976). Within the bilayer many enzyme proteins control transmembrane ionic fluxes (Dhalla et al, 1978). In addition, the sarcolemma contains receptor sites for drugs and hormones (Dhalla et al, 1977). The role of the glycocalyx in the regulation of heart function is not well understood but it has been suggested that it may regulate the supply of contractile dependent calcium (Howse et al, 1970; Shine et al, 1971; Langer, 1975; 1978).

Cell Surface Material and the Negative Charge

Many techniques have been employed to study the composition of this surface layer with a variety of mechanisms of interaction with and specificity for the different components of the glycocalyx (Martinez-Palomo, 1970). Cell coats containing carbohydrates and proteins are thought to play an important role in membrane permeability (Eylar, 1965). Bennett (1963) suggested the preponderance of acidic mucopolysaccharides composing the cell surface material could be responsible for the selective permeability to ions exhibited by muscle cells. Langer (1978) felt in this regard that the phospholipid and oligosaccharide

components of the sarcolemma were involved in selective binding of cations rather than in regulation of beat to beat ionic translocations.

It is thought the fixed negative charge at the cell surface (Elul, 1967) may be responsible for myocardial cation binding (Hajdu and Leonard, 1976; Langer, 1978). This concept has led investigators to attempt to identify anionic sites with a variety of cytochemical probes (Nicolson, 1973; Langer et al 1976; Frank et al, 1977; Langer, 1978; Takeo et al, 1979; Matsukubo et al, 1979). An early study by Howse et al (1970) in myocardial cells of both invertebrate and vertebrate species demonstrated that ruthenium red and colloidal iron (CI) stained the surface material confirming the polyanionic character of this membrane component. A number of investigators have used colloidal iron (Mowry, 1958) to visualize negatively charged sites on the surface of whole tissues, isolated cells and cell membranes (Gasic and Berwick, 1963; Yardley and Brown, 1965; Benedetti and Emmelot, 1967; Howse et al, 1970; Nicolson, 1973;Zacks et al, 1973a; 1973b; Langer et al, 1976; Frank et al, 1977; Langer, 1978; Matsukubo et al, 1979). Information obtained to the present indicates CI is bound to negatively charged membrane sites which, in theory, could include acid mucopolysaccharides, acidic amino acids and phospholipids (Blanquet and Loiez, 1973). A study by Zacks et al (1973a), using CI and ruthenium red, indicated the surface coat was composed of glycoproteins containing a substantial number of carboxyl groups. The surface coat, termed

the "external lamina" in this investigation, was found to have a high content of acidic amino acids, chiefly glutamine and asparginine. In this study, as well as a subsequent one (Zacks et al, 1973b), neuraminidase failed to alter the structure or ruthenium red staining characteristics of the surface layer. Another study (Vandenburgh et al, 1974) on the chemical composition of isolated rat skeletal sarcolemma, concluded that the external lamina substance, extractable by lithium diiodosalycilate (LIS) was enriched in acidic amino acids which might account for its polyanionic character.

In contrast, almost total loss of CI stain in rat liver membranes (Benedetti and Emmelot, 1967) and myocardium (Langer et al, 1976; Frank et al, 1977) following treatment of these membrane preparations with neuraminidase was interpreted to mean that CI was binding to n-acetylneuraminic acid (sialic acid). Biochemical analysis of the same preparations however, indicated only 60-70% of the total membrane sialic acid was sensitive to neuraminidase, suggesting that some sialoglycoproteins were insensitive to or inaccessable to both CI stain and neuraminidase action. Nicolson (1973), in a study on red cell ghosts, reported hydrolysis of 90% of membrane sialic acid by neuraminidase with a concommitant decrease in CI staining to 85% of control. Yardley and Brown (1965) and Mareel et al (1976) reported CI staining was sensitive to hyaluronidase, suggesting some CI is bound to sulfated mucopolysaccarides.

Sialic Acid and Calcium Binding

Sialic acid has been shown to bind calcium preferentially (Jaques et al, 1977) and is believed to control the supply of this cation at the sarcolemma (Shine et al, 1971; Langer et al, 1975; Langer, 1976; 1978). Thus desialation of sarcolemmal membranes by neuraminidase would be expected to be associated with a decrease in calcium binding. Regulation of membrane permeability to calcium by sialic acid has been reported (Langer et al, 1976; Langer, 1978; Frank et al, 1977). These studies demonstrated removal of sialic acid by neuraminidase resulted in an increase in calcium exchangability and entry of lanthanum, normally impermeant, into the cell. Neuraminidase inhibition of calcium binding has also been reported (Shlatz and Marinetti, 1972; Limas, 1977), however, the extent of inhibition and therefore of the relative importance of sialic acid as a calcium binding moiety is controversial. Limas (1977) found neuraminidase treatment of rat sarcolemma had a relatively small effect (-8.2%) on calcium binding, whereas treatment with proteolytic enzymes resulted in a marked decrease of 40-50%. Feldman and Weinhold (1977) found calcium binding associated with a purified lipoprotein component of the rat sarcolemma. In view of these findings the extent to which neuraminidase sensitive CI staining of rat myocardium can be said to represent calcium binding sites is unresolved.

Lanthanum as a Cytochemical Marker of Calcium Binding Sites

Since the report of Sanborn and Langer (1971) describing the uncoupling of excitation from contraction in cardiac muscle by

lanthanum, this trivalent cation has been used to demonstrate possible calcium binding sites. Shine and Langer (1976) found displacement of radioactive calcium by lanthanum was accompanied by a concommitant decrease in contractile force. Limas (1977) found lanthanum inhibited calcium binding to low affinity sites only. Ruthenium red on the other hand inhibited calcium binding to both high and low affinity sites. The high affinity site, which was unaffected by lanthanum in this investigation, was associated with a protein peak which bound radioactive ATP suggesting the high affinity site might represent a calcium ATPase. Recently, a study by Takeo et al (1979) demonstrated binding of lanthanum to the sarcolemmal membrane accompanied by a significant decrease in both calcium binding and calcium ATPase activities. The apparant discrepancies in the two studies as regards the effect of lanthanum on calcium ATPase have not been explained.

Cytochemical studies on the sites of lanthanum binding and modification of the staining pattern by enzymatic treatments have yeilded conflicting results. Langer and Shine (1976) demonstrated lanthanum was bound to the basement membrane and suggested that the binding sites were most probably negatively charged carboxyl groups. Others, however, consider membrane phospholipids to be lanthanum binding sites. Doggenweiler and Frenk (1965) described lanthanum staining in a variety of tissues and suggested this cation might be bound to phosphatidylserine and phosphatidylethanolamine. In addition, Lesseps (1967) removed

lanthanum staining material by treatment with phospholipase C. Overton (1968) described lanthanum binding in intestinal brush border cells which was sensitive to trypsin and pronase.

Lanthanum binding to myocardial sarcolemma does displace calcium bound to the membrane as evidenced by the rapid decline in contractile force when this cation is present (Sanborn and Langer, 1971; Shine and Langer ,1976). However, a wide variety of enzymatic modifications have been effective in reducing or eliminating membrane sites to which lanthanum is bound (Doggenweiler and Frenk, 1965; Lesseps, 1967; Overton, 1968).

Identification of calcium binding sites by the use of cationic probes clearly has not resolved the controversy surrounding the identity of calcium binding biomolecules. Apparent contradictions in the literature pertaining to other properties of cell membranes, in addition to calcium binding, may be either species or tissue related or, in the case of isolated membranes a result of the characteristics of the preparation itself. Vanderburgh et al (1974) suggested isolation procedures could be expected to modify weak covalent bonds binding some components of polyphasic membrane systems. The influence of isolated membrane vesicle sidedness on the properties of membrane bound enzymes, as well as their degree of leakiness, has been documented by Besch et al (1976) in a study of the vectorial properties of the Na^+-K^+ATPase of canine cardiac sarcolemma. It has become evident that comparison of the sarcolemma isolated by different procedures is only meaningful if the preparations being

compared are well characterized.

Membrane Isolation Procedures

Many protocols for the isolation of membrane fractions enriched in sarcolemma have been reported. In general, procedures follow two basic patterns or in some cases a combination of both. One group utilizes a comparatively non-invasive fractionation on a sucrose density gradient. Kidwai et al (1971) were amongst the first to apply this method to the isolation of cardiac sarcolemma. The sarcolemmal fraction thus obtained was characterized by a high specific activity of Na^+-K^+ ATPase, a putative sarcolemmal marker enzyme (DePierre and Karnovsky,1973). Electron microscopy of the fraction revealed its vesicular morphology and the absence of identifiable contamination by other cellular organelles or myofilaments. Various modifications of the procedure of Kidwai et al (1971), including the addition of mild extractions in either KCl or KCl combined with pyrophosphate, have been adopted by other investigators (Hui et al, 1975; St. Louis and Sulakhe, 1976; Heller and Harary, 1977; Bers, 1979; Misselwitz et al, 1979). In addition to Kidwai et al (1971), St. Louis and Sulakhe (1976), Bers (1979), and Misselwitz et al (1979) all reported high specific activity of the Na^+-K^+ ATPase in the isolated sarcolemmal fraction. However, the degree of sensitivity of this enzyme to ouabain, a cardiac glycoside, was variable. Such differences in ouabain sensivity have been attributed to both the orientation (sidedness) of sarcolemmal vesicles, as well as to their relative leakiness (Besch et al,

1976). Hui et al (1975), Misselwitz et al (1979) and St. Louis and Sulakhe (1976) copurified the sarcolemmal fraction and adenylate cyclase activity which is believed by some to be present exclusively at the plasma membrane. Both Heller and Harary (1977) and Bers (1979) labelled the plasma membrane prior to isolation with radioactive iodine. Heller and Harary (1977) subsequently recovered radioactivity in the sarcolemmal fraction, while Bers (1979) demonstrated parallel distribution of the Na^+-K^+ ATPase and radioactive label. Misselwitz et al (1979) labelled the isolated fraction with concanavalin A, a plant lectin with a known specificity for alpha-D-mannose and alpha-D-glucose. As membrane carbohydrate assymetry is absolute (Rothman and Lenard, 1977), this histochemical study demonstrated not only the presence of a glycocalyx but also the orientation of the majority of sarcolemmal vesicles.

A second type of procedure for isolation of the sarcolemma from cardiac muscle cells combining repeated differential centrifugation, hypotonic shock and extraction of the membrane pellet with salts such as KCl and/ or LiBr has been adopted by other workers (Sulakhe and Dhalla, 1971; McNamara et al, 1974; Dhalla et al, 1977). McNamara (1974) reported this preparation contained a high specific activity of the ouabain sensitive Na^+-K^+ ATPase. Electron microscopy indicated minimal contamination with other cellular organelles or myofilaments (Anand et al, 1977). The derivation of the sarcolemma from the cardiac cell membrane was demonstrated in a study by Takeo et al

(1979). In this report, membranes isolated by the hypotonic shock-LiBr procedure from hearts perfused with lanthanum revealed the presence of electron dense deposits at the surface of membranes in the isolated sarcolemmal fraction. Lanthanum staining also revealed the majority of the vesicles were labelled external to the bilayer indicating the preparation was essentially oriented right side out with an intact glycocalyx.

It would appear therefore, that the many protocols for isolation of cardiac sarcolemmal membranes yeild a fraction enriched in sarcolemma possessing some properties which are qualitatively similar. There are, however, discrepancies with regard to other characteristics. Some preparations contain a calcium dependent ATPase (McNamara et al, 1974; Dhalla et al, 1976) while others report a calcium stimulated, magnesium dependent enzyme (St.Louis and Sulakhe, 1976). In addition controversy exists regarding sarcolemmal calcium binding with some reporting a passive phenomenum (Dhalla et al, 1977; 1979) whilst others conclude that binding is enhanced by ATP (St. Louis and Sulakhe, 1976; Limas, 1977; Mas-Oliva et al, 1979).

From the foregoing it is obvious that the studies on isolated membranes have been hampered by a variety of factors. It is now documented that species related differences in enzymatic properties of heart sarcolemma are present (Lamers et al, 1979). As well, the isolation procedures required to obtain purified fractions probably do alter sarcolemmal properties (Vandenburgh et al, 1974; Besch, 1976). It is generally accepted that

characterization of isolated membranes is complicated by the difficuty in obtaining pure preparations devoid of other cellular contaminants. Finally, sarcolemma may be oriented in either the right side out or inside out conformations or in a heterogeneous mixture of both (Besch et al, 1976). Right side out and inside out conformation are referenced to the sidedness of the membrane in the intact cell.

It would appear appropriate, therefore, to study preparations from the same species isolated by at least two of the generally accepted protocols and to characterize the fractions both biochemically and cytochemically to determine if the observed differences are primarily a result of one or a combination of the above factors.

METHODS AND MATERIALS

Neuraminidase (Cl. perfringens) Type V, phospholipase C (Cl. welchii), trypsin (bovine pancreas) and cationized ferritin (horse spleen) were obtained from Sigma. Trypsin inhibitor (egg white) was purchased from Calbiochem. $^{45}CaCl_2$ was supplied by New England Nuclear.

Male Sprague-Dawley rats (Biobreeding, Ottawa,Canada) weighing 250-300 grams were maintained with access to standard rat chow and water ad libitum in a controlled environment.

Isolation of Rat Heart Sarcolemma

Hearts removed from rats immediately following decapitation, as well as those which had been perfused as described later, were placed in ice cold buffer and the sarcolemmal fraction was isolated by two different methods.

Method I

In this method for the isolation of heart sarcolemma, the hypotonic shock- LiBr procedure described by McNamara et al, (1974) as modified by Dhalla et al, (1977) was employed.

Hearts were washed in ice cold 10mM Tris-HCl, pH 7.4 containing 1 mM ethylenediamine-tetraacetate (EDTA) and only the ventricular tissue free of atria and connective tissue was used for the isolation of sarcolemma. The ventricles were minced finely with scissors in the above buffer and the resulting material was then homogenized in 10 vol (w/v) for 1 min in a Waring blender at speed 5. The homogenate was filtered through four layers of guaze to remove coarse material and cell debris .

The filtrate was centrifuged at 1000 g for 10 min. The pellet was suspended in 10 vol of 10 mM Tris-HCl, pH 7.4, and stirred for 15 min. Following centrifugation at 1000 g for 10 min the pellet was resuspended in 10 vol of 10mM Tris-HCl, pH 8.0, stirred for 15 min and centrifuged at 1000 g for 10 min. This step was repeated in 10mM Tris-HCl, pH 7.4. The washed particles were suspended in 20 vol Tris-HCl, pH 7.4, containing 0.4 M LiBr, stirred for 30 min and centrifuged at 1000 g for 10 min. The pellet was suspended in 10 mM Tris-HCl, pH 7.4, stirred for 15 min and centrifuged at 1000 g for 10 min. The sarcolemma enriched pellet was further extracted in 10 mM Tris-HCl, containing 0.6 M KCl, pH 6.8, for 15 min and centrifuged at 1000 g for 10 min. The pelleted sarcolemma was washed for 15 min in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 and centrifuged at 1000 g for 10 min. The final pellet was suspended in 1 mM Tris-HCl, pH 7.0, and employed in the subsequent investigations on the sarcolemma. All isolation steps were carried out at 4 C and fresh membranes were used for all determinations.

Method II

In this method for the isolation of sarcolemma the discontinous sucrose gradient procedure of Kidwai et al (1971) was adopted as described below.

Hearts were washed in ice cold 0.25 M sucrose and the atria and connective tissues were removed. The ventricles, minced finely with scissors and suspended in 10-20 vol 0.25 M sucrose, were homogenized in a Polytron PCU I at half the maximal speed

for 15 sec and full speed for 15 sec. The homogenate was filtered through 2 layers of guaze and layered on top of a discontinuous gradient composed of 8 mls each of 39%, 49% and 82% sucrose (w/w). The homogenate was centrifuged for 90 min at 55,000 g in a SB 116 swinging bucket rotor in a Beckman B 60 ultracentrifuge. The F1 band at the interface of the 0.25 M and 39% sucrose layers was removed with a pasteur pipette and diluted with 0.25 M sucrose. This fraction was centrifuged at 90,000 g for 20 min in an A-211 rotor. In some studies the F2 fraction at the interface of the 39% and 49% sucrose layers was also removed with a pasteur pipette and further isolated in the same manner as described for the F1 fraction. The pellets were suspended in 0.25 M sucrose and used for further analysis. All steps were carried out at 4 C. Mostly fresh membranes were used for subsequent determinations while the remainder of the membrane fraction was frozen at -70 C for later use.

Biochemical Procedures:

Enzyme treatments of sarcolemma

Sarcolemmal membranes which had been isolated by method I were incubated for 20 min at 37 C in 50 mM Tris-HCl, pH 7.4, containing 20 mM KCl to which neuraminidase (.31 U/mg protein) or trypsin (100 ug/mg protein) had been added. Incubation with phospholipase C (1 U/mg protein) was carried out in the same buffer in the presence of 2 mM $CaCl_2$. Neuraminidase treatment was terminated by cooling in ice. Trypsin treatment was terminated by the addition of 2-3x trypsin inhibitor. Phospholipase C treatment

was terminated by the addition of 2 mM EDTA.

Enzyme assays

Adenosine triphosphatase enzyme activities of the membranes isolated by Method I and II were studied by suspending 40-50 ug/ml and 10-20 ug/ml of membrane protein respectively in a medium containing 50 mM Tris-HCl, pH 7.4. For magnesium dependent Na^+-K^+ATPase activity the incubation medium contained 0.1 M NaCl, 10 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA. The activity of Ca^{2+}ATPase was studied in the presence of 4 mM $CaCl_2$ and the activity of Mg^{2+} ATPase was studied in the presence of 4 mM $MgCl_2$, 1 mM EDTA. The difference between the Mg^{2+} dependent Na^+-K^+ATPase activity and the Mg^{2+}ATPase activity was taken to be the Na^+-K^+ATPase. Ca^{2+}/Mg^{2+}ATPase (calcium stimulated, magnesium dependent) was studied in the presence of 4 mM $CaCl_2$ and 4 mM $MgCl_2$. Membranes were preincubated for 3 min at 37 C and the reaction was started by the addition of 4 mM Tris ATP. The reaction was terminated by the addition of 1 ml of 12% ice cold trichloroacetic acid. Phosphate liberated in the above incubations was determined according to the procedure of Taussky and Shorr (1953). Protein was estimated by the method of Lowry et al (1951). Ouabain sensitivity of the Na^+-K^+ ATPase was determined by studying the activity of this enzyme in the presence of 2 mM ouabain.

Calcium binding

For the study of calcium binding activity in membranes isolated by either Method I or Method II, two different incubation media were employed. Incubation medium A consisted of

50 mM Tris-HCl, pH 7.4. Membrane protein (0.15 mg/ml), suspended in medium A, was preincubated for 3 min at 37 C. The reaction was started by the addition of 100 ul $^{45}CaCl_2$ at a concentration of either $1x10^{-4}$ or $1.25x10^{-3}$ M. The reaction was terminated by millipore filtration after 5 min (Sulakhe and Dhalla, 1971). Blanks, without protein, were incubated under the identical conditions described above. Samples of the filtrate (100 ul) were dissolved in 10 mls of Beckman Ready-Solv scintillation cocktail and vials were counted in a Beckman liquid scintillation counter. Incubation medium B contained 25 mM Tris maleate (pH 6.8), 10 mM $MgCl_2$, 100 mM KCl, 2.5 mM Tris ATP. (Sulakhe et al, 1976). Membrane protein (0.15 mg/ml) was added to medium B. The mixture was preincubated for 3 min at 37 C and the reaction was started by the addition of $1x10^{-4}$ M $CaCl_2$. In these experiments the calcium binding activity in Method II membranes was also studied by incubating these membranes in the absence of ATP. The reaction was terminated by millipore filtration after 5 min and filtrates were sampled and counted as described above. Calcium binding for method II membranes was obtained by subtracting the activity in the absence of ATP from the value obtained in the presence of ATP.

Sialic Acid Determination

Sialic acid released by either neuraminidase (neuraminidase sensitive) or by acid hydrolysis (total) was determined by the thiobarbituric acid assay of Warren (1963). For measurement of the neuraminidase sensitive component of sarcolemma, membranes

were incubated with the enzyme (.31 U/mg protein) in an assay medium consisting of 50 mM Tris-HCl, pH 7.4, containing 20 mM KCl for 20 min at 37 C. For total sialic acid content determination, untreated membranes were washed and hydrolyzed in 0.1 N H_2SO_4 for 1 hr at 80 C to release total sialic acid (Cook, 1976). The enzyme treated Method I membranes were also hydrolyzed as described above to determine neuraminidase insensitive sialic acid content.

Cytochemical Procedures

For routine morphological examination of membranes, the pellet was fixed in 2% glutaraldehyde in phosphate buffer for 2 hrs. The pellet was then washed for 1 hr in phosphate buffer containing 0.1 M sucrose. Post fixation was performed in 1% osmium tetroxide for 1 hr at 4 C. The membranes were dehydrated in a graded alcohol series and embedded in Epon 812 (Luft, 1961). Thin sections were cut on a Porter-Blum ultramicrotome placed on Formvar coated grids, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a Zeiss EM 9 electron microscope.

Colloidal iron staining

Sarcolemmal membranes (I and II) were suspended in 2% glutaraldehyde in phosphate buffer, for 45 min at 4 C. Following centrifugation, the pellet was washed in the same buffer, followed by two changes (5 min each) of 12% acetic acid and resuspended in freshly prepared colloidal iron solution, pH 1.2-1.3, for a period of 1 hr at room temperature (Gasic and

Berwick, 1963). Following staining, the membranes were centrifuged, washed in four changes (3 min each) of 12% acetic acid and postfixed in 1% osmium tetroxide for 1 hr at 4 C. The membranes were processed for conventional electron microscope examination as described above.

In a second study sarcolemmal membranes were fixed and stained with CI in the manner described earlier. These membranes were applied directly to Formvar coated grids and examined in the electron microscope (Benedetti and Emmelot, 1967; Nicolson, 1973). This procedure yeilds a surface view of the colloidal iron distribution on the sarcolemma.

Lanthanum and ferritin staining

Two procedures were employed to study the distribution of lanthanum and ferritin in the isolated rat heart sarcolemma. In the first study, isolated sarcolemmal membranes (Methods I and II) were suspended in lanthanum (1 mM) or ferritin (0.1 mg/ml) for 5 min. Following centrifugation, the membranes were washed, fixed in 2% glutaraldehyde containing either lanthanum (1 mM) or ferritin (0.1 mg/ml) for 1 hr. Membrane pellets were then washed, postfixed with 1% osmium tetroxide and prepared for electron microscopic study as described earlier.

A second study was conducted to assess what differences would be observed if lanthanum or ferritin were perfused through the isolated rat heart prior to isolation of the sarcolemmal fraction.

Perfusion of Hearts with Lanthanum or Ferritin

Rats were sacrificed by decapitation and their hearts were excised and placed in ice cold oxygenated Krebs-Henseleit (K-H) solution for ferritin perfusions or ice cold oxygenated HEPES buffer for lanthanum perfusions (Sanborn and Langer, 1970). Atrial, fat and connective tissues were removed and the hearts were perfused according to the method of Langendorf. Before the start of the experiments hearts were equilibrated for 15 min by perfusion with either K-H solution containing (mM): NaCl, 120; $NaHCO_3$, 25; KCl, 4.8; KH_2PO_4, 1.2;$MgSO_4$, 1.25; $CaCl_2$, 1.25; and glucose, 8.6;pH 7.4, or HEPES solution containing (mM): NaCl, 145;KCl, 4; $CaCl_2$, 2.5;$MgCl_2$, 1.0; glucose, 5.56 and N_2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES),3.0;pH 7.4. Both perfusion solutions were maintained at 37'C. K-H solution was continuously gassed with 95% O_2 and 5%CO_2 and HEPES solution with 100% O_2. All hearts were stimulated electrically at 300 beats/min using a Phipps and Bird square wave stimulator. The coronary perfusion rate was 7.8-8.0 ml/min. The contractile activity was monitored through a Beckman recorder employing a force displacement transducer. At the end of the equilibration period of 15 min, either ferritin (0.1mg/ml) or lanthanum (1 mM) was added to the K-H and HEPES buffer respectively. After 5 min, a 1 min washout with the appropriate buffer was performed. Following exposure to either lanthanum or ferritin, sarcolemmal isolation was performed according to either Method I or Method II. Following isolation, membranes were fixed and processed routinely for electron microscopic study.

Statistical Analysis

The results obtained comparing enzyme and calcium binding activities as well as sialic acid content (neuraminidase sensitive and total) of membranes isolated by either Method I or Method II were analyzed using the t statistic for 2 means. Results obtained for enzyme and calcium binding activities of Method I membranes following enzymatic treatment with either trypsin, phospholipase C or neuraminidase were analyzed using the paired t statistic. Comparison of enzyme and calcium binding activities as well as sialic acid content (neuraminidase sensitive and total) of the F1 and F2 fractions of Method II membranes were analyzed using a paired t statistic. All values are the mean $\pm$ S.E. of at least 4-6 experiments. Statistical significance was taken as $p<.05$.

RESULTS

Comparative Studies of the Method I and Method II Membranes

Morphology and enzyme characteristics of the membrane

Morphological appearance of the membranes isolated by hypotonic shock-LiBr treatment (Method I) and sucrose density gradient (Method II) is shown in Fig 1 and the data on enzymatic activities have been summarized in Table 1.

It has been reported earlier that the membrane fraction obtained by the hypotonic shock-LiBr method (I) originates from the myocardial cell membrane and has been found to be free of contaminants such as myofilaments, mitochondria and nuclei. Marker enzyme studies of this membrane preparation also revealed minimal contamination (3-5%) with other cytoplasmic organelles (Anand et al, 1977). The preparation was found to consist of vesicles of variable size and shape. The outer surface of most vesicles was not smooth due to the presence of a fuzzy material external to the plasma membrane (Fig 1a), which has been shown to represent the cell surface material (Takeo et al, 1979).

Membranes isolated by the sucrose density gradient method are believed to be derived from the myocardial sarcolemma (Kidwai et al, 1971). Electron microscopic examination of the fraction has revealed no identifiable contamination of the preparation by other cellular organelles or myofilaments (Kidwai et al, 1971). Membranes were present as small spherical structures (Fig 1b) without any readily apparent surface material.

The Method I membranes in the present study showed a high

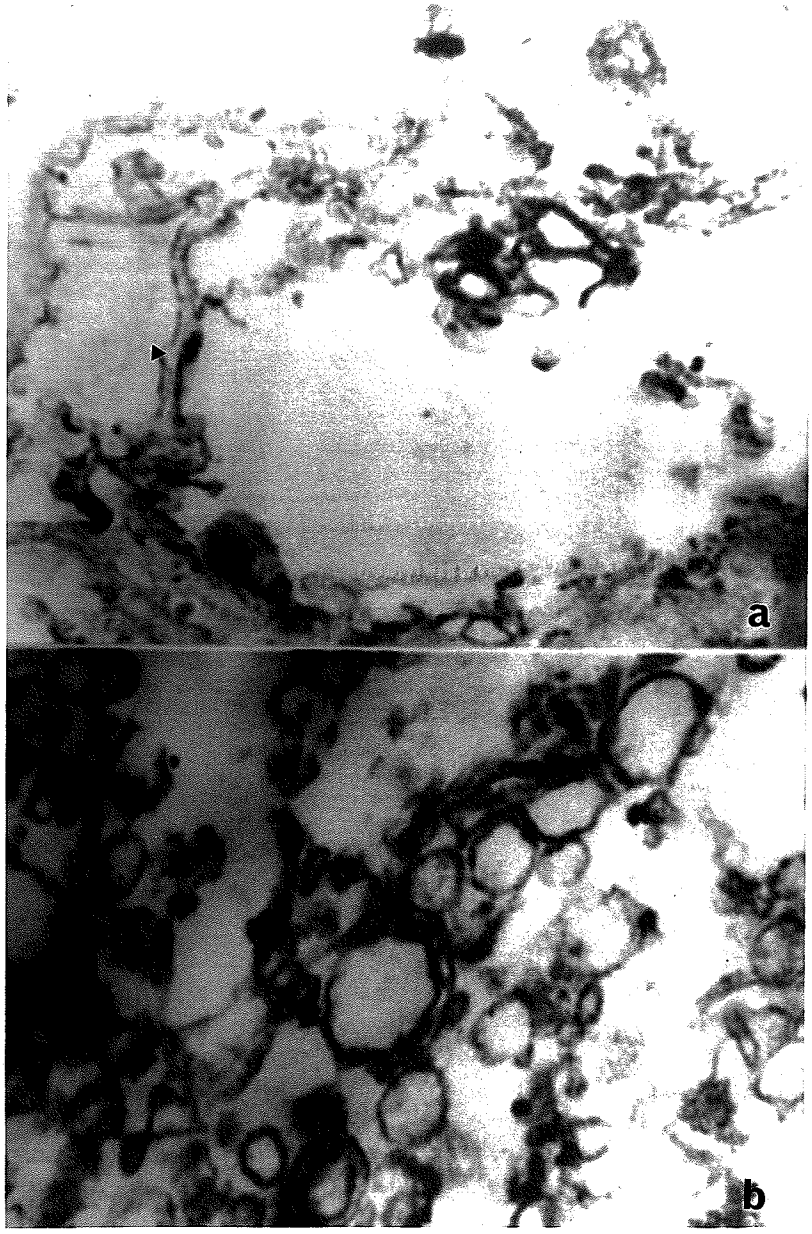

Fig. 1a. Sarcolemmal membranes isolated by Method I. Cell surface material (arrow) from 0.1-0.3 u in thickness is seen adhering to the unit membrane. x31,000

Fig. 1b. Sarcolemmal membranes isolated by Method II. Vesicles appear to have no cell surface material. x90,000

Table I. Enzyme Activities of Rat Heart Sarcolemma Isolated by Two Different Procedures.

Enzyme Activity (umoles Pi/mg protein per hr)	Isolation Procedure I	Isolation Procedure II
Mg^{2+} ATPase	28.60 ± 1.66	104.37 ± 6.26*
Ca^{2+} ATPase	32.20 ± 1.44	115.47 ± 9.22*
Na^{+}- K^{+} ATPase	13.20 ± 0.81	17.75 ± 3.70
Na^{+}- K^{+} ATPase + 2 mM ouabain	3.19 ± 0.57 (76%)	16.12 ± 1.94* (9%)

Na^{+}- K^{+} ATPase activity was measured in the presence of 1 mM EDTA, 4 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl. Mg^{2+} ATPase was determined in a medium containing 1 mM EDTA and 4 mM $MgCl_2$. Ca^{2+} ATPase activity was measured in the presence of 4 mM $CaCl_2$. All other conditions were as described in Methods.

(%) percent inhibition in the presence of 2 mM ouabain.

Each value is the mean ± S.E. of at least 4-8 experiments.

* Significantly different from the Procedure I value ($p < 0.05$)

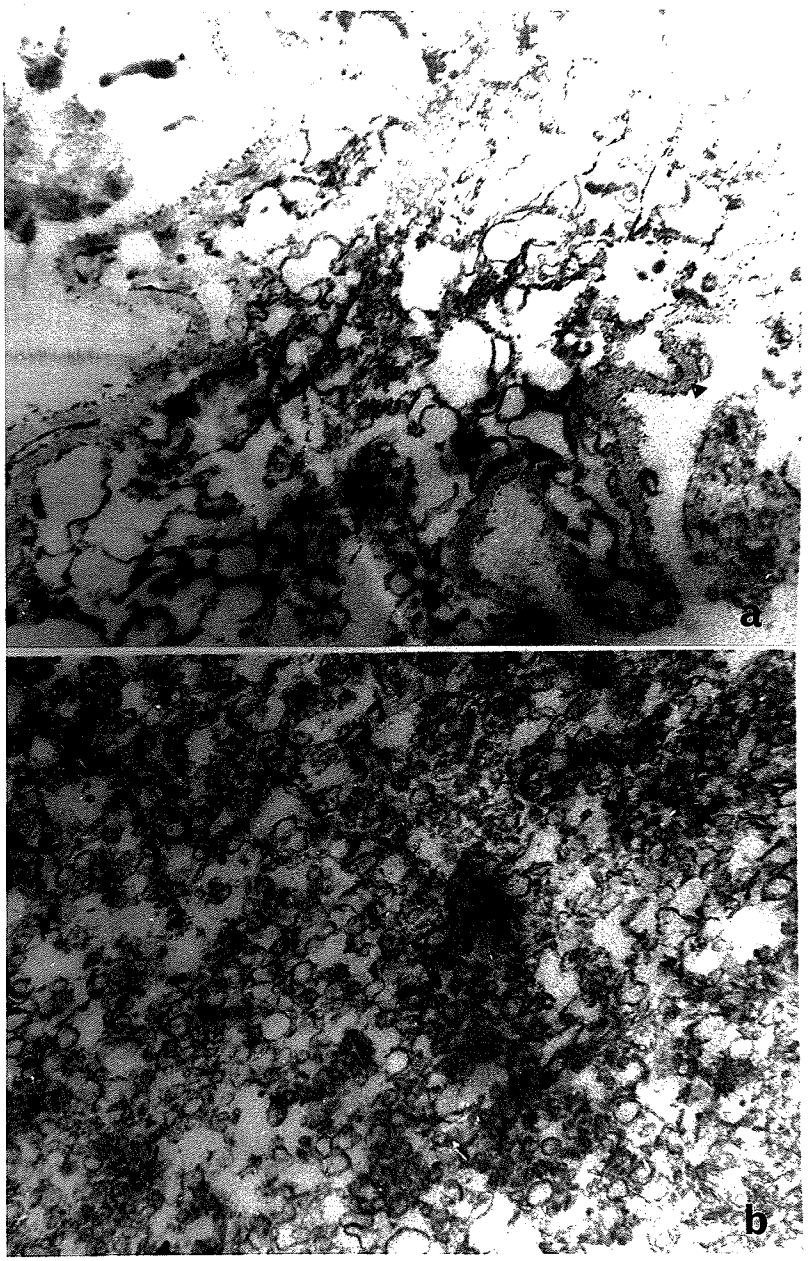

Fig. 2a. Sarcolemmal membranes (Method I) stained with CI. Stain is seen adhering to the cell surface material (arrow). x30,000

Fig. 2b. Sarcolemmal membranes (Method II) stained with CI. x28,000

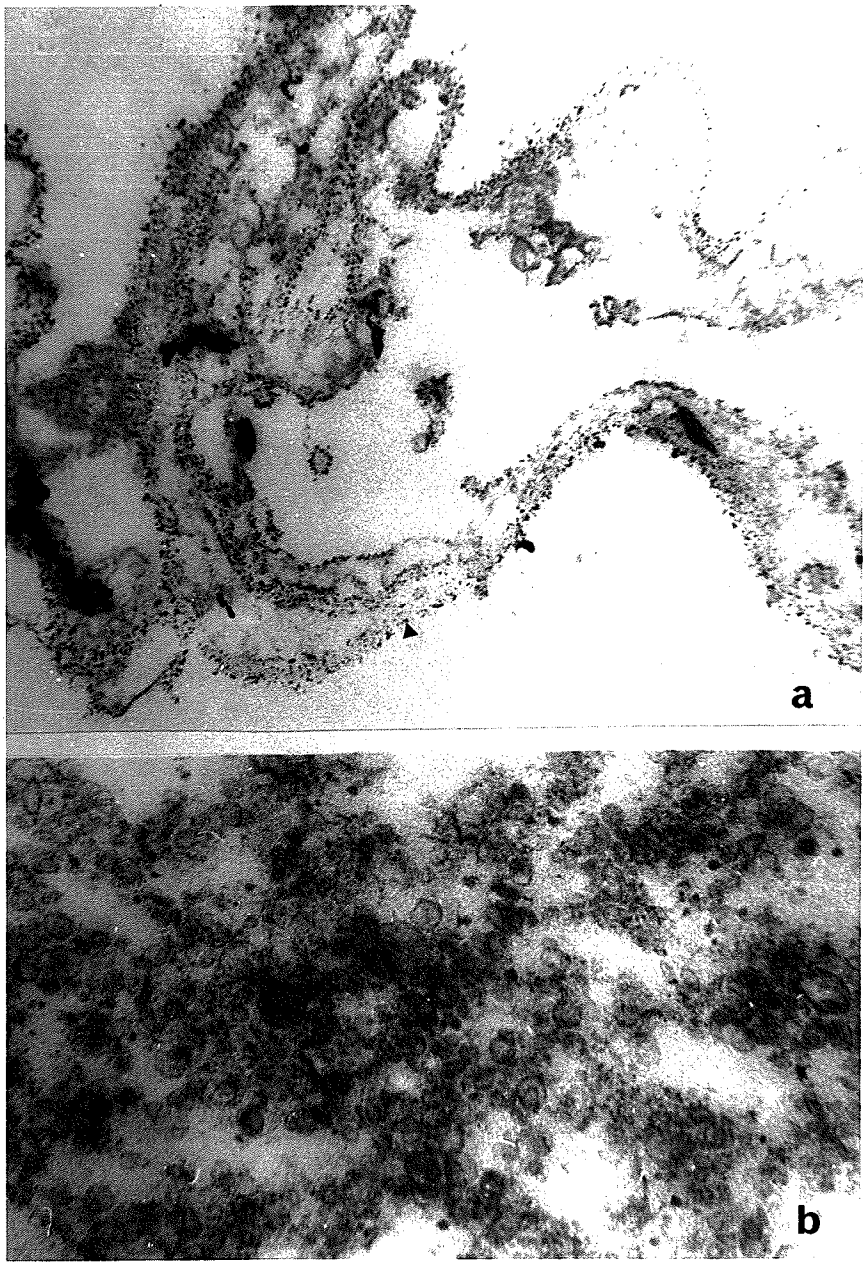

Fig. 3a. Sarcolemmal membranes (Method I) stained with lanthanum. Lanthanum staining of the cell surface material (arrow). x28,000

Fig. 3b. Sarcolemmal membranes (Method II) stained with lanthanum. x28,500

appeared to be located at the plasma membrane. In comparing hypotonic shock-LiBr membranes to the preparation discussed here it is readily apparent that both morphology and CI staining patterns differ fundamentally.

Lanthanum staining

Both preparations were studied with respect to their lanthanum staining characteristics. Two protocols were followed for both membrane preparations. In the first approach, isolated membranes were incubated with lanthanum (1 mM) prior to fixation. Lanthanum staining of isolated Method I membranes was uniform with most of the probe adhering to the fuzzy surface material (Fig 3a). In contrast, lanthanum staining of isolated Method II membranes was sparse (Fig 3b) displaying a pattern similar to that observed in the presence of CI. In the second approach, hearts were perfused with lanthanum (1 mM) for 5 min. As expected the contractile activity was reduced to zero in a matter of seconds (Fig 4).

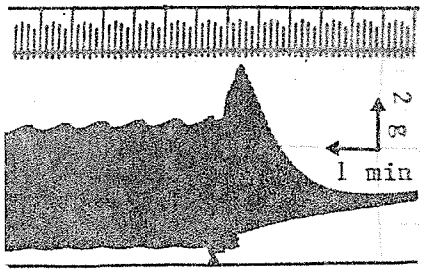

FIg. 4. Effect of La^{3+} on contractile force of the isolated rat heart. Arrow indicates addition of La^{3+} to the perfusion medium.

When these perfused hearts were employed for the isolation of the sarcolemmal fraction by the hypotonic shock-LiBr method the

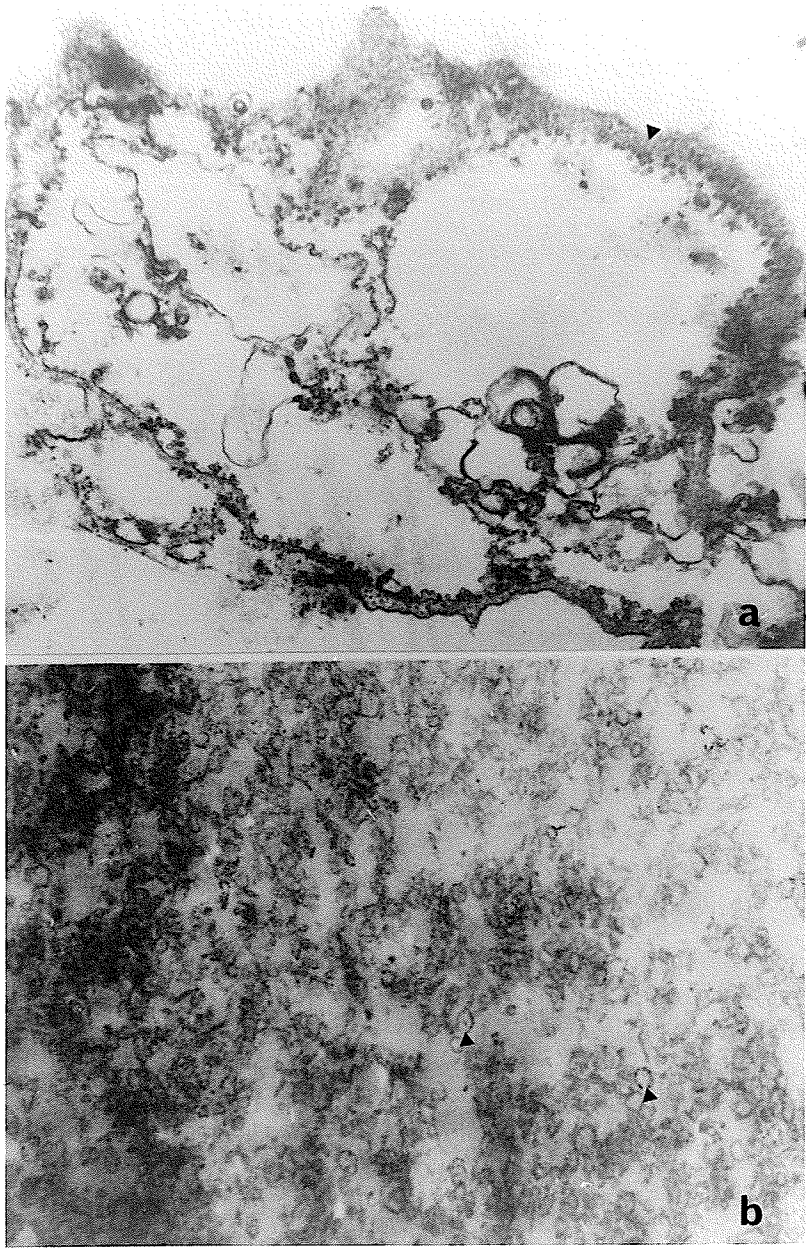

Fig. 5a. Sarcolemmal membranes (Method I) from hearts perfused with lanthanum prior to isolation. Lanthanum staining of the cell surface material (arrow). x21,000

Fig. 5b. Sarcolemmal membranes (Method II) from hearts perfused with lanthanum prior to isolation. Occasional staining of the plasma membrane (arrows). x27,500

lanthanum staining was observed to be restricted to the surface material (Fig 5a). Membranes which were isolated by the sucrose density gradient method (II) following perfusion with lanthanum, showed occasional staining of membrane vesicles to both the inner and outer aspects of the sarcolemma (Fig 5b).

Ferritin staining

As in the lanthanum study, both types of membranes were incubated with ferritin (0.1 mg/ml) prior to fixation. As well, hearts were perfused with this cationized iron storage protein for 5 min and the sarcolemmal fraction was subsequently isolated by both procedures. Perfusion with ferritin rapidly reduced contractile force to zero (Fig 6).

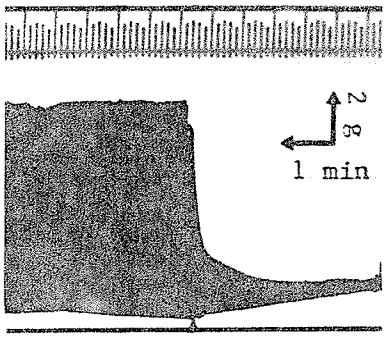

Fig. 6. Effect of ferritin on contractile force of the isolated perfused rat heart. Arrow indicates addition of ferritin to the perfusion medium.

Ferritin was bound to the cell surface material in a uniform pattern in membranes which had been isolated by the hypotonic shock-LiBr procedure prior to staining (Fig 7a). In contrast, sucrose gradient membranes showed little ferritin binding (Fig 7b) and the staining pattern of Method II membranes was qualitatively as well as quantitatively similar to that observed

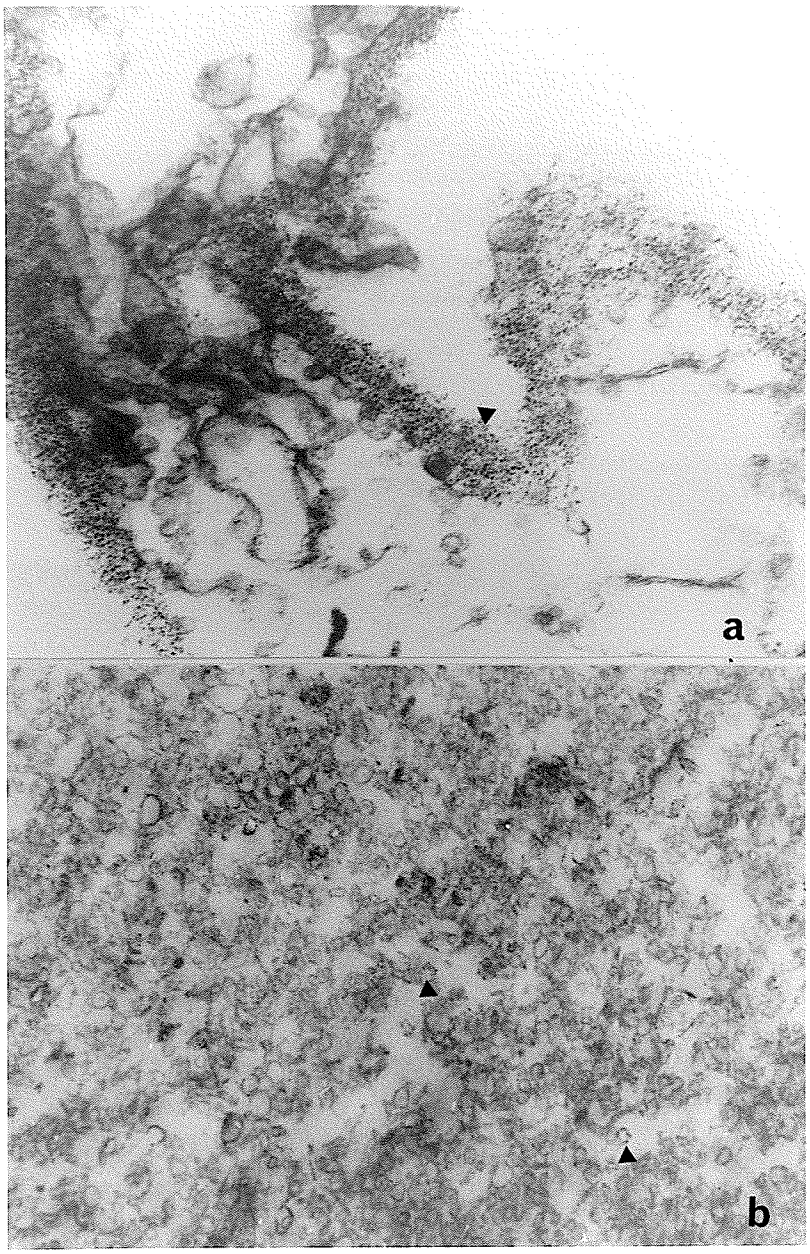

Fig. 7a. Sarcolemmal membranes (Method I) stained with ferritin. Ferritin staining of the cell surface material (arrow). x78,000

Fig. 7b. Sarcolemmal membranes (Method II) stained with ferritin. Occasional staining of the plasma membrane (arrows). x28,000

previously with both CI and lanthanum.

Calcium binding

Calcium binding characteristics of sarcolemmal membranes isolated by Method I and Method II were investigated employing two different incubation media in the presence or absence of ATP. The results of these studies have been summarized in Table 2.

Membranes were incubated in 50 mM Tris-HCl, pH 7.4, and 0.1 mM calcium in the absence of ATP (incubation medium A). Method II membranes bound 4.42 nmoles Ca^{2+}/mg protein per 5 min or 18 % of the value obtained under identical conditions for Method I membranes. As values were obtained in the absence of ATP, this binding was termed non-specific calcium binding.

In another set of experiments both membrane preparations were tested for calcium binding activity in the presence of ATP by using an incubation mixture consisting of 25 mM Tris maleate (pH 6.8), 100 mM KCl, 10 mM $MgCl_2$, 2.5 mM Tris ATP and 0.1 mM $CaCl_2$ (incubation medium B). The binding observed under these conditions was termed ATP dependent binding. The difference in calcium binding values in the presence or absence of ATP was taken to represent the ATP dependent component of calcium binding. Calcium binding to Method I membranes was inhibited in the presence of ATP and therefore showed no ATP dependent component. Approximately 90% of sarcolemmal calcium binding to Method II membranes in incubation medium B was found to be ATP dependent with a mean value of 15.7 ± 2.18 nmoles Ca^{2+}/mg protein per 5 min.

Table 2. Calcium Binding Activity of Rat Heart Sarcolemma Isolated by Two Different Procedures.

	Calcium Binding ($nmoles\ Ca^{2+}/mg$ protein per 5 min)	
	Incubation Medium	
Sarcolemmal Isolation Procedure	A	B
I	24.5 ± 1.96*	5.1 ± 0.89*
II	4.4 ± 0.58*	17.5 ± 2.24* (with ATP) 1.8 ± 0.79 (without ATP)

Isolation procedures as described in Methods.

Medium A: 50 mM Tris-HCl, pH 7.4, 0.1 mM $CaCl_2$. Medium B: 25 mM Tris maleate, pH 6.8, 10 mM $MgCl_2$, 100 mM KCl, 2.5 mM Tris ATP, 0.1 mM $CaCl_2$.

* Significantly different for the same fraction in Medium A or B and for both fractions in either Medium A or B. ($p < 0.05$)

The data obtained from cytochemical stainings as well as calcium binding studies clearly indicated differences in the surface negative charge as well as calcium binding activity of the membranes isolated by Methods I and II. Since sialic acid accounts for a significant amount of surface negativity (Gottschalk, 1966;Eylar et al, 1962;Shlatz and Marinetti, 1972) as well as calcium binding (Long and Mowat, 1971), it was decided to compare the sialic acid content in the two membrane preparations. The results of this study have been summarized in Table 3.

Sialic acid content and neuraminidase sensitivity

In this series of experiments sarcolemmal membranes (Method I and II) were incubated with neuraminidase (0.31 U/mg protein) as described in methods. Neuraminidase treatment of Method I membranes released 25.4 ± 2.70 nmoles sialic acid /mg protein while Method II membranes released 0.87 ± 0.50 nmoles sialic acid/mg protein. Subsequent hydrolysis of the neuraminidase treated Method I membranes released a further 14.9 ± 0.02 nmoles sialic/mg protein. Acid hydrolysis was performed on untreated Method I and Method II membranes. This treatment yeilded a total sialic acid content of 40.4 ± 3.14 nmoles/mg protein for Method I membranes and 1.01 ± 0.66 nmoles/mg protein for Method II membranes. Sialic acid content reported here for Method I membranes is similar to that reported for rat myocardial sarcolemma by McConnaughey et al (1979).

Because of the somewhat surprising finding that the

Table 3. Sialic Acid Content and Neuraminidase Sensitivity of Rat Heart Sarcolemma Isolated by Two Different Procedures.

Sialic Acid (nmoles/mg protein)	Isolation Procedure I	Isolation Procedure II
Released by Neuraminidase (.31 U/mg protein)	25.4 ± 2.70	0.87 ± 0.50*
Released by Acid Hydrolysis (Total)	40.4 ± 3.14	1.01 ± 0.66*

All conditions as described in Methods.

Each value is the mean ± S.E. of at least 4 experiments.

* Significantly different from the Procedure I value. ($p < 0.05$)

membranes obtained in the FI fraction of the sucrose density gradient procedure as described by Kidwai et al (1971) contained almost no sialic acid, it was decided to examine some properties of the F2 fraction on the sucrose density gradient. However, it should be noted Kidwai et al (1971) have claimed that this fraction is derived from the sarcoplasmic reticulum although no marker enzyme studies of this fraction were reported. Thus, we compared F1 and F2 fractions with respect to their sialic acid content (neuraminidase and total) as well as enzymatic and calcium binding activities. The data from these studies have been summarized in Table 4. The activities of Ca^{2+}ATPase and Mg^{2+}ATPase were elevated in the F2 fraction with values 37% and 90% higher respectively than those found in the F1 fraction. The F2 fraction contained no detectable Na^{+}-K^{+}ATPase activity either in the presence or absence of 2 mM ouabain. ATP dependent calcium binding was similar in both fractions, that is, 15.69 ± 2.18 nmoles/mg protein per 5 min for the F1 fraction and 12.90 ± 3.52 nmoles/mg protein per 5 min for the F2 fraction. However, in the F2 fraction a substantial component of calcium binding (11.92 ± 0.83 nmoles/mg protein per 5 min) was ATP independent. This activity was minimal in the F1 fraction (1.83 ± 0.79 nmoles/mg protein per 5 min). The F2 fraction showed considerably more sialic acid with only a nominal increase in the neuraminidase sensitive component.

At this point it became quite apparent that out of the three membrane fractions (Method I, Method II F1 and Method II F2)

Table 4. Comparison of Enzyme Activities, Calcium Binding Activity and Sialic Acid Content of Two Fractions Derived from the Sucrose Density Gradient Procedure.

Activity Measured	Membrane Fraction	
	FI	FII
I. Enzyme Activity (umoles Pi/mg protein per hr)		
Mg^{2+} ATPase	104.37 ± 6.26	197.99 ± 19.04*
Ca^{2+} ATPase	115.47 ± 9.22	157.95 ± 9.23*
Na^{+}- K^{+} ATPase	17.75 ± 3.70	N.D.*
II. Calcium Binding (nmoles Ca^{2+}/mg protein per 5 min)		
i) ATP Dependent	15.69 ± 2.18	12.90 ± 3.52
ii) ATP Independent	1.83 ± 0.79	11.91 ± 0.83*
III. Sialic Acid (nmoles/mg protein)		
i) Neuraminidase Sensitive	0.87 ± 0.50	2.16
ii) Total	1.01 ± 0.66	12.06

All conditions as described in Methods.

All values are the mean ± S.E. of 4 experiments. (with the exception of sialic acid data for FII)

* Significantly different from the value for the FI fraction ($p < 0.05$).

examined thus far only Method I membranes exhibited the presence of cell surface material, as evidenced by cationic staining patterns, as well as sialic acid and Na^+-K^+ATPase activity. It was therefore decided to conduct further cytochemical and biochemical studies only on the Method I membranes.

Enzyme Treatment of Method I Membranes

The membranes isolated by hypotonic shock-LiBr method were treated separately with trypsin, phospholipase C and neuraminidase. These enzyme treated membranes were then examined for their CI staining, enzyme activities and calcium binding.

Morphology and CI staining

Trypsin treatment of membranes affected their morphological appearance as well as subsequent binding of CI to the cell surface material. The results of a typical experiment are shown in Fig 8a and b. In sectioned material sarcolemmal membranes were represented largely as membranous sheets (Fig 8a) rather than by vesicles, as was the case in the control preparation. CI staining of the cell surface material was considerably reduced after trypsin treatment as can be seen in the sectioned material (Fig 8a) as well as in surface view (Fig 8b). Membranes from the untreated preparation seen in both sectioned and surface view (Fig 9 a and b), on the other hand, revealed a high density of CI binding sites and the distribution of CI in this view was fairly uniform.

The effects of phospholipase C treatment on the density and distribution of CI are shown in Fig 10 a and b. In sectioned

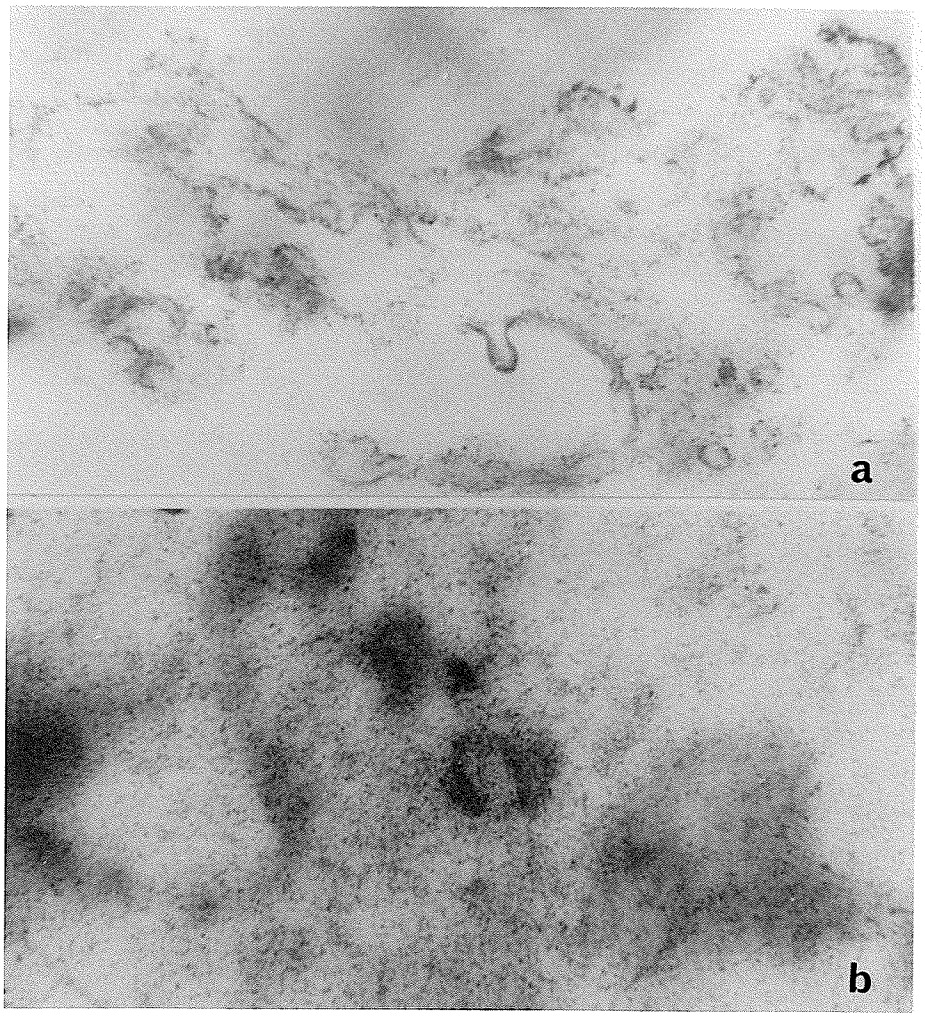

Fig. 8. Effects of trypsin treatment (100 ug/mg protein) on CI staining of the sarcolemma.
a) Sectioned membranes: marked reduction in CI staining as well as poor membrane definition due to this treatment. x23,000
b) Membranes in surface view: uniform reduction in CI staining. x70,000

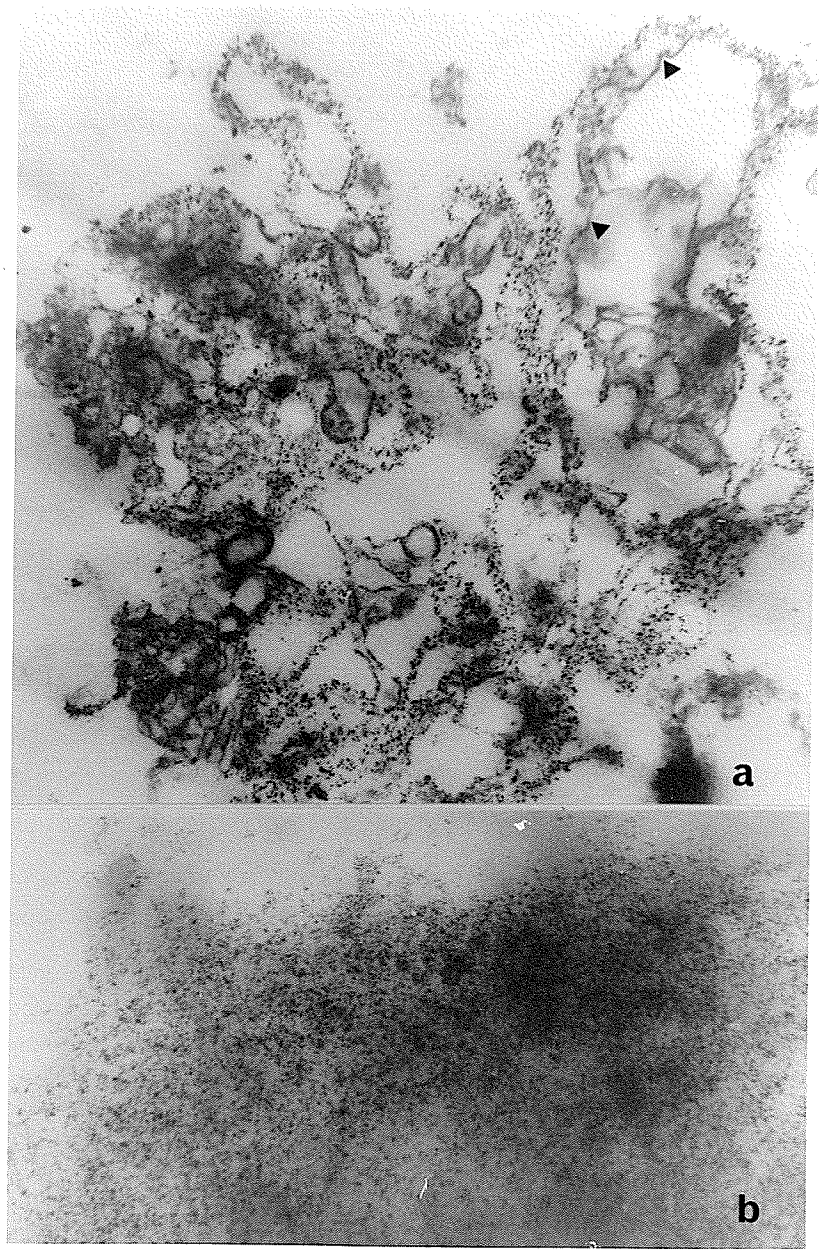

Fig. 9. Colloidal iron staining of the isolated rat heart sarcolemma.
a) Sectioned membranes: CI particles are mostly seen in the fuzzy layer. This layer, at places, is separated from the unit membrane (arrows). x27,000
b) Membranes in surface view: CI forms a dense pattern over the membrane surface. x78,000

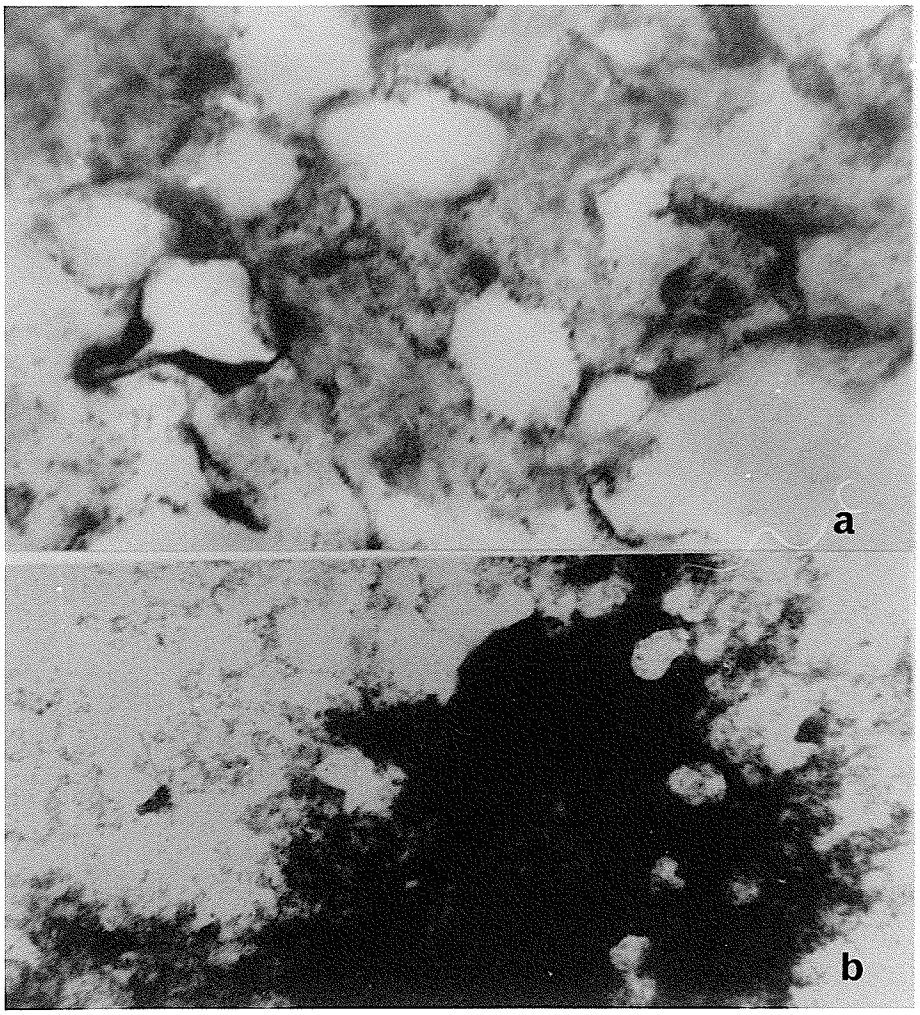

Fig. 10. Effects of phospholipase C treatment (1 U/mg protein) on CI staining.
a) Sectioned membranes: marked reduction in CI staining as well as size of the vesicles is apparent. x58,000
b) Membranes in surface view: marked decrease in CI staining. x35,000

material, a morphological alteration in sarcolemmal membranes, manifested chiefly by a substantial reduction in the surface material as well as in the size of vesicles was apparent. In addition, CI staining of the surface material in the sectioned membranes as well as in the surface view was markedly reduced.

Neuraminidase treatment of the membranes had no apparent effect on their morphological appearance (Fig 11 a and b). CI staining of the surface material after neuraminidase treatment was consistently less than in controls. In surface view the loss of CI staining was found to be patchy in appearance and the remaining CI particles were seen to aggregate in a random fashion.

Enzyme assays

Hypotonic shock-LiBr membranes (I) were also analyzed for sarcolemmal enzyme activities after treatment with either trypsin, phospholipase C or neuraminidase. The results are shown in Table 5. Treatment of membranes with trypsin resulted in an increase in the specific activities of both Mg^{2+}ATPase and Ca^{2+}ATPase by 22% and 26% respectively over values observed in the untreated (control) preparation. In contrast, Na^{+}-K^{+}ATPase activity was reduced 90% by this treatment. Phospholipase C caused a slight depression in Mg^{2+}ATPase and Ca^{2+}ATPase but these changes were not significant. However, a marked reduction (85%) in the activity of Na^{+}-K^{+}ATPase was observed following phospholipase C treatment. Treatment of membranes with neuraminidase resulted in a comparable reduction (30-40%) in the

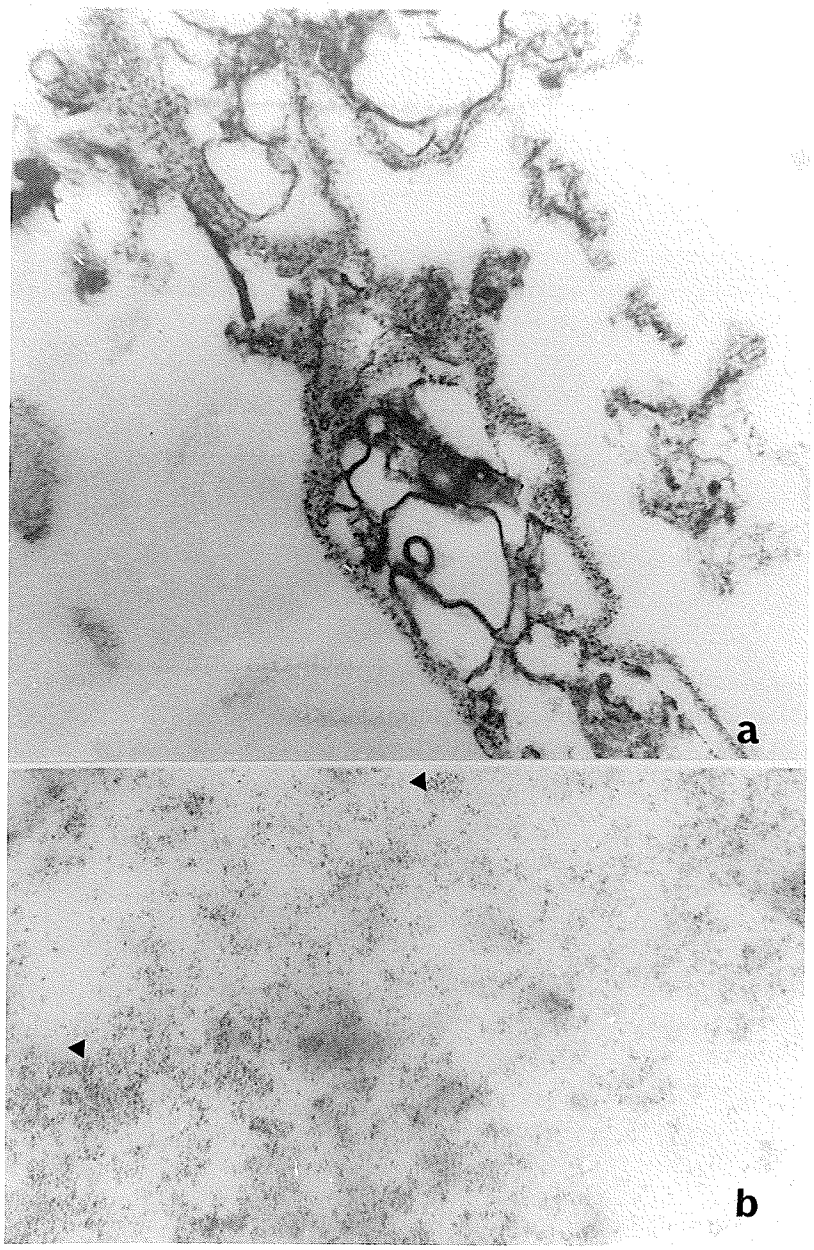

Fig. 11. Effects of neuraminidase treatment (0.31 U/mg protein) on CI staining of the sarcolemma.
a) Sectioned membranes: In this view only marginal reduction in CI staining is apparent. x26,000
b) Membranes in surface view: A definite reduction in CI staining as well as random aggregation (arrows) of CI particles. x72,500

Table 5. Effects of Trypsin, Phosphlipase C and Neuraminidase on the Activities of Mg^{2+} ATPase, Ca^{2+} ATPase and Na^{+}- K^{+}ATPase of Rat Heart Sarcolemma.

Enzyme Activity (umoles Pi/mg protein per hr)	Treatment			
	Control	Trypsin	Phospholipase C	Neuraminidase
Mg^{2+} ATPase	32.3 ± 1.15	39.3 ± 3.57*	30.4 ± 2.03	21.5 ± 1.87*
Ca^{2+} ATPase	35.5 ± 2.13	44.9 ± 2.80*	32.6 ± 1.40	23.0 ± 1.62*
Na^{+}- K^{+} ATPase	14.4 ± 1.06	1.6 ± 0.88*	2.2 ± 0.29*	8.0 ± 0.90*

Na^{+}- K^{+} ATPase activity was measured in the presence of 1 mM EDTA, 4 mM $MgCl_2$, 100 mM NaCl and 10 mM KCl. Mg^{2+} ATPase was determined in a medium containing 1 mM EDTA and 4 mM $MgCl_2$. Ca^{2+} ATPase activity was determined in the presence of 4 mM $CaCl_2$. All other conditions were as described in Methods.

Each value is the mean ± S.E. of 4-8 experiments.

* Significantly different from the control value ($p < 0.05$).

activities of all three enzymes and these changes were statistically significant.

Calcium binding

Rat heart sarcolemma is reported to have both high and low affinity calcium binding sites with affinity constant values of $2x10^{-5}$ and $5.6x10^{-2}$ respectively (Limas, 1977). In this study calcium at concentrations of 0.1 mM and 1.25 mM was used to analyze the binding characteristics of the Method I membranes both before and after treatment with trypsin, phospholipase C and neuraminidase. The results are shown in Table 6. Control membranes bound 24.5 ± 1.96 nmoles Ca^{2+}/mg protein per 5 min in the presence of 0.1 mM calcium. Increasing the calcium concentration to 1.25 mM resulted in a ten fold increase in calcium binding to 248 ± 14.34 nmoles Ca^{2+}/mg protein per 5 min.

Calcium binding in the presence of 0.1 mM calcium was reduced by trypsin and neuraminidase treatments but was unaffected by treatment with phospholipase C. Furthermore, trypsin was found to be more effective than neuraminidase in reducing calcium binding at this concentration. However, calcium binding activity of the membranes in the presence of 1.25 mM calcium was reduced with any of the membrane perturbing agents employed in this study with the order of potency starting from the most effective agent being trypsin > neuraminidase > phospholipase C.

Table 6. Effects of Trypsin, Phospholipase C and Neuraminidase on Calcium Binding by Rat Heart Sarcolemma.

Ca^{2+} concn. (mM)	Calcium Binding (μmoles Ca^{2+}/mg protein per 5 min)			
	Control	Trypsin	Phospholipase C	Neuraminidase
0.1	24.5 $\pm$ 1.96	7.2 $\pm$ 0.41*	22.1 $\pm$ 1.52	14.4 $\pm$ 0.77*
1.25	248.5 $\pm$ 14.34	126.4 $\pm$ 8.58*	169.1 $\pm$ 2.75*	142.4 $\pm$ 20.12*

Assay medium as described in Methods.

Each value is the mean $\pm$ S.E. of 4-6 experiments.

* Significantly different from the control value ($p < 0.05$).

DISCUSSION

It is known that the plasma membrane constitutes the major barrier between the intracellular and extracellular compartments. In vitro studies of plasma membranes, including the myocardial sarcolemma, have provided insights into the functional properties of the cell membrane. However, the impact of such studies on physiological interpretation has been limited by the conflicting reports which have appeared in the literature (Dhalla et al; 1977). This situation has been exacerbated by the seemingly impossible task of obtaining a purified membrane fraction from complex mammalian cells. Only in studies of red cells, which contain no intracellular membrane systems, has the problem of fraction purity been satisfactorily resolved. These studies indicate the functional assymetry of cell membranes is accompanied by a structural assymetry. The latter is more or less absolute for membrane carbohydrates and proteins, and relative with respect to membrane phospholipids (Rothman and Lenard, 1977). It is pertinent to study the effects of structural alteration on established characteristics and functional properties of the membrane. The study of membrane morphology, surface charge and the orientation of membranous vesicular fractions derived from the cell membrane is amenable to cytochemical techniques, whilst enzyme activities and calcium binding are best studied using biochemical procedures. The combined biochemical and cytochemical approach of the present investigation, employing two different procedures for membrane

isolation, provides an integrated and comprehensive picture regarding some sarcolemmal characteristics.

In the first part of this study, sarcolemma isolated by a sucrose density gradient technique (Kidwai et al, 1971) was compared to that obtained the hypotonic shock-LiBr method (McNamara et al, 1974;Dhalla et al, 1977).

Failure of CI to stain the fraction obtained by the sucrose density gradient method (II) suggested this sarcolemma differed fundamentally from that obtained by the procedure of McNamara et al (1974). The two fractions were morphologically distinct, in as much as Method I membranes exhibited a visible call surface material and in Method II membranes no such structure was evident. Possible explanations which could account for the difference in the CI staining pattern of the two preparations include: a) complete absence of cell surface material: b) a fraction oriented predominantly in the inside out conformation thus rendering the surface material inaccessable to CI or: c) derivation of this fraction from a different membrane system or from a specialized region of the sarcolemma. The complete absence of surface material was indicated by the lack of CI staining, however, with this technique alone the presence of cell surface material facing the interior of the vesicle could not be ruled out for Method II membrane vesicles. This possibility was preempted by the sialic acid data. Biochemical analysis of this preparation to determine sialic acid content, revealed that FI sarcolemmal fraction was insensitive to neuraminidase and

surprisingly contained minimal amounts of sialic acid when these membranes were hydrolyzed with 0.1 N H_2SO_4 to determine total sialic acid content. This finding suggested that the Method II membranes did not possess a sialic acid containing glycoprotein layer. The possibility that sialic acid might have been selectively removed from the surface material during fractionation and that the glycoprotein layer was still present but oriented in the inside out conformation was ruled out by the perfusion experiments employing lanthanum and ferritin. These cationic probes are known to bind to the different components of the sarcolemmal membrane complex. This technique, utilizing lanthanum as a probe, was adopted by Takeo et al (1979) to demonstrate the presence of cell surface material as well as to ascertain that the sarcolemmal fraction isolated by the hypotonic shock-LiBr procedure (McNamara et al, 1974) originated from the myocardial membrane.

Therefore, based on the cytochemical evidence with respect to the staining characteristics of Method II sarcolemmal membranes in the presence of either CI, lanthanum or ferritin, whether applied directly to the isolated fraction or perfused through the heart prior to isolation, it is reasonable to conclude that the glycoprotein enriched surface layer is most likely absent in the FI band. This would account for the absence of sialic acid in this fraction.

The orientation of isolated membrane fractions derived from the myocardial sarcolemma and its effect on the functional

properties displayed by these preparations, has been studied extensively by Besch et al (1976), St.Louis and Sulakhe (1978), Jones et al (1979). Besch and associates (1976) felt treatment of sarcolemmal preparations with salt solutions at high concentrations might unmask Na^+-K^+ATPase activity by rendering the vesicles "leaky" and therefore passively permeable to ions, ATP and ouabain. It would appear that this may in fact be so in the sarcolemmal fraction isolated by the procedure of McNamara et al (1974). Electon microscopic evidence (Anand et al, 1977;Takeo et al, 1979; Matsukubo et al,1979) and neuraminidase sensitivity (Matsukubo et al, 1979;Takeo et al, 1980) indicate this fraction is predominantly oriented in the right side out conformation. This contention is supported by the data of the current investigation. The high specific activity of the Na^+-K^+ATPase found in the present study would seem to indicate the presence of membranes permeable to both sodium and ATP as both the binding site for sodium and the catalytic site are located at the cytoplasmic side of the sarcolemma. The high sensitivity of this enzyme to ouabain in this preparation (II) may also be due to right side out orientation of the sarcolemma vesicles. Both the cytochemical and biochemical evidence in the present study indicates these conclusions are valid for membranes isolated by the hypotonic shock- LiBr procedure (McNamara et al, 1974).

In contrast, sarcolemmal membranes isolated by the sucrose density gradient procedure (Kidwai et al, 1971) displayed a high specific activity of the Na^+-K^+ATPase accompanied by a low

sensitivity to ouabain (10%). These findings suggest the preparation consists of predominantly inside out vesicles leaky to ions and ATP but with low permeability characteristics to ouabain. The orientation of the vesicles in Method II membranes may also explain the extremely high values for the Mg^{2+}ATPase and Ca^{2+}ATPase in this fraction. In this context, calcium efflux has been shown to be ATP dependent (Sulakhe et al, 1976) in myocardial sarcolemma. However, the possibility that the high specific activity of Mg^{2+} and Ca^{2+} ATPases might be the result of activation of these enzymes following the loss of the glycoprotein layer cannot be ruled out. In fact, this type of activation was noted in Method I membranes following trypsin treatment in the present study. In this regard, activation of the (Mg^{2+},Ca^{2+}) ATPase following treatment of the sarcolemmal preparation with low concentrations of trypsin has also been reported (St.Louis and Sulakhe, 1978).

The calcium binding characteristics of the two preparations in the present study were diverse. The hypotonic shock-LiBr fraction (I) bound calcium in a non-specific manner and this activity was inhibited in the presence of magnesium and ATP. Marked (50%) inhibition of calcium binding in this preparation in the presence of 0.1 mM calcium when 2 mM Mg^{2+}-ATP was present has been reported previously (Dhalla et al, 1976). The current investigation showed 79% inhibition of calcium binding in the presence of high (10 mM) magnesium and ATP (2.5 mM). Since magnesium is known to compete for calcium binding sites and to

uncouple excitation from contraction (Shine and Langer, 1976;Feldman and Weinhold, 1977;Bers and Langer, 1979) and maximal ATP hydrolysis in the presence of either calcium or magnesium in Method I membranes is reported to occur at concentrations of 4-8 mM (Anand et al, 1977) the observed inhibition of calcium binding in the presence of a high (10 mM) magnesium concentration is not surprising. It should be noted, however, that ATP (2 mM) itself also inhibited calcium binding to Method I membranes in the study of Dhalla et al (1976).

Sucrose density gradient membranes (II) in contrast, displayed calcium binding which was enhanced in the presence of magnesium and ATP. Non specific binding shown by Method I membranes was minimal in this fraction. The calcium binding characteristics of Method II membranes resemble those reported for sarcolemma by Sulakhe et al (1976). This preparation of highly purified sarcolemmal membranes (Sulakhe et al, 1976) has been shown to have a ATP dependent calcium pump located at the cytoplasmic side of the membrane which displays ATP dependent calcium binding. In fact, the incubation medium (B) used in the present investigation, is the same as that used by St.Louis and Sulakhe (1976) to study calcium binding in their preparation. In addition, ATP supported calcium binding in rat (Limas, 1977) and rabbit (Mas-Oliva et al, 1979) sarcolemma has been reported previously. Both of these studies included magnesium in the incubation medium.

The preliminary study comparing the FI and F2 bands in

Method II membranes has provided some insight into the nature of the sucrose density gradient preparation. It would appear that the F2 band contains some sarcolemmal vesicles as evidenced by the finding of sialic acid, considered to be a sarcolemmal component (Bers, 1979), in this fraction. Furthermore, lack of neuraminidase sensitivity would seem to indicate that these vesicles are probably oriented inside out. This is supported by other work from our laboratory (unpublished) showing the presence of adenylate cyclase activity in the F2 band. Interestingly, the F2 band contained a higher adenylate cyclase activity than the FI band and that activity was not enhanced in the presence of epinephrine or sodium fluoride. Although the origin of the F2 fraction from this initial work cannot be ascertained with any certainty, the fraction seems to represent an admixture of membranes derived from the sarcoplasmic reticulum and the sarcolemma.

At any rate, the comparative data on Method I and II membranes obtained under similar experimental conditions suggest that membrane isolation in itself could result in alteration of the structural properties of the sarcolemma which might account for observed variations in the functional properties of this membrane which have appeared in the literature. Since Method I membranes showed characteristics relatively more similar to the "in situ" conditions, vis a vis right side out orientation and presence of cell surface material, these membranes were subjected to further in depth examination to study the structure-function

relationship.

It is well documented that CI at pH 1.2-1.8 employed in the present study binds to the cell surface (Gasic and Berwick, 1963;Yardley and Brown, 1965; Benedetti and Emmelot, 1967;Howse et al, 1971;Nicolson, 1973;Langer et al, 1976;Frank et al, 1977;Matsukubo et al, 1979). Although there is still no agreement with respect to the chemical nature of the sites binding CI it is generally accepted such sites are negatively charged. The present study demonstrates the presence of cell surface material as well as negative charge in the rat heart sarcolemma isolated by the hypotonic shock-LiBr procedure (McNamara et al, 1974). Two different cytochemical protocols were employed using CI as a common probe. Furthermore, a comprehensive picture regarding the distribution of negative charges could be constructed by combining the results of CI staining from the sectioned as well as the surface view of the membranes. The high density of CI particles observed in the surface view was most likely due to superimposition of particles scattered throughout the surface coat and on the plasma membrane. The two tier distribution of CI in the sectioned material is comparable to that reported in rat and rabbit myocardium (Langer et al, 1976;Frank et al, 1977;Langer, 1978). In addition, the reported separation of the basement membrane from the unit membrane seen in rat hearts perfused with calcium free medium for a prolonged time (Tomlinson et al, 1974;Frank et al, 1977) may in fact correspond to the occurance of a wide gap between the two layers observed in the

isolated sectioned membranes. In this regard, it should be noted that isolation of sarcolemma in this study involved homogenization and washing in the presence of 1 mM EDTA, a known chelator of calcium. It seems reasonable to conclude that the rat heart sarcolemma isolated by the method of McNamara and associates (1974) as modified by Dhalla et al (1977) contains cell surface material as well as negative charge in two mutually separable layers external to the unit membrane and the results of the cytochemical study are qualitatively similar to CI staining reported in cultured heart cells and myocardium (Frank et al, 1977).

Mowry (1958) considered CI to be specific for acidic polysaccharides at pH 1.1-1.3 but warned of non-specific staining at pH 1.4 or higher. The CI stain has been used for the cytochemical demonstration of membrane bound sialic acid (Benedetti and Emmelot, 1967;Gasic and Berwick, 1965; Nicolson, 1973;Langer et al, 1976;Frank et al, 1977;Mareel et al, 1976; Matsukubo et al, 1979) as well as hyaluronic acid and sulfated mucopolysaccharides (Yardley and Brown, 1965;Mareel et al, 1976). It has been pointed out that the positively charged colloid can, in theory, bind not only to carboxyl and sulfate groups of mucopolysaccharides, but also to carboxyl groups of acidic amino acids and phosphate groups of phospholipids (Blanquet and Loiez, 1973). The results of this study support this contention in as much as pretreatment with either trypsin, phospholipase C or neuraminidase reduced CI staining . Reduction in CI staining

following proteolytic digestion has been demonstrated in red cell membranes (Nicolson, 1973) and the treatment has been reported to release membrane bound sialic acid as a sialoglycopeptide in red cells (Makela et al, 1960;Nicolson, 1973; Triplett and Carraway, 1972), rat liver membranes (Benedetti and Emmelot, 1967) and rat hepatoma cultures (Baumann and Doyle, 1979). However, specific removal of over 60% of membrane bound sialic acid by neuraminidase in the present study was not as effective in reducing CI staining as was trypsin. This result could be anticipated if CI binds not only to sialic acid but to membrane proteins. If this were to be the case, removal of membrane peptides and sialoglycopeptides with trypsin would have an additive effect in eliminating CI binding sites. Furthermore, lanthanum which is believed to displace calcium at the cell surface (Sanborn and Langer,1970) has been found associated with a surface layer which was sensitive to trypsin and pronase (Overton,1968). Others have reported phospholipid involvement in the binding of lanthanum to the cell surface (Doggenweiler and Frenk, 1965;Lesseps, 1967). In view of these observations as well as those of the current investigation it is difficult to restrict negatively charged sites binding CI and lanthanum to sialic acid as has been suggested. The results of the present study and those of previously reported studies, further strengthen the view that the surface negative charge is due to a mosaic of chemical groups.

The results of the current investigation on calcium binding

properties of trypsin and neuraminidase treated sarcolemma indicate that proteins and glycoproteins may also play a major role in both the high affinity and low affinity calcium binding pools. The existence of at least two calcium binding pools situated at the cell membrane in myocardial sarcolemma (Limas, 1977), liver (Schlatz and Marinetti, 1972), and erythrocytes (Duffy and Schwarz, 1972) has been reported. Limas (1977) found trypsin dramatically reduced calcium binding to the rat heart sarcolemma whereas phospholipases and neuraminidase were without significant effect on this parameter. In contrast, we observed a significant reduction in membrane calcium binding at both high and low affinity sites following treatment with trypsin or neuraminidase and at low affinity sites following phospholipase C. The reason for these differences between the two studies is not clear at present. However, reduction in the number of available cation binding sites following phospholipase C and neuraminidase treatments indicated by the reduction in calcium binding was accompanied by a corresponding decrease in CI staining of the treated membranes. Sialic acid has been identified as a molecule which will bind calcium in a preferential manner (Jaques et al, 1977), thus the lack of any significant effect of neuraminidase treatment on sarcolemmal calcium binding in Limas' study (1977) is intriguing, as neuraminidase treatment, in that investigation, resulted in the loss of 75% of membrane bound sialic acid. Furthermore, the failure of phospholipase C to significantly reduce calcium

binding at low concentrations of calcium (0.1 mM) would suggest that phospholipids may contribute more to the low affinity, high capacity calcium binding pool.

Neuraminidase has been reported by a number of workers to substantially prevent subsequent binding of CI in a variety of tissues (Benedetti and Emmelot, 1967;Nicolson, 1973;Langer et al, 1976;Frank et al, 1977). While neuraminidase always reduced staining in our preparation, it never eliminated it. Benedetti and Emmelot (1967), Langer et al (1976) and Frank et al (1977) all reported 60-70% of membrane sialic acid was released by neuraminidase and at the same time almost total abolition of CI staining was noted in these studies. Nicolson (1973) reported hydrolysis of 90% of membrane sialic acid by this enzyme. In that study however, reduction in CI staining paralleled the loss of sialic acid from the membrane. In the present study removal of 63% of membrane sialic acid resulted in a definite reduction in staining although the exact percentage could not be ascertained from this essentially qualitative approach. It appears therefore that the remaining CI binding sites either are not sialic acid or if they are, they possess alpha-ketosidic linkages not cleaved by neuraminidase under our experimental conditions. Such neuraminidase resistant bonds have been reported by Drzeniek (1973). The effects of trypsin and phospholipase C, two membrane perturbing agents, on three sarcolemmal enzymes are not unexpected as loss of proteins or lipids not only alters the microenvironment of the membrane but also may have a direct

effect on the enzyme molecules themselves (Coleman, 1973). Lipid requirements for all three enzymes studied in the current investigation have been reported (Roelofsen and Van Deenen, 1973). As enzymes are proteins, proteolytic digestion would be expected to exert profound effects on enzyme function. The rather uniform depression of Mg^{2+}ATPase, Ca^{2+}ATPase and Na^{+}-K^{+}ATPase activities following neuraminidase treatment would suggest that either sialic acid forms an essential part of all three enzymes or that reduction in the density and distribution of surface charge following this treatment plays a role in the regulation of the activities of these membrane bound enzymes. The latter view is consistent with a recent report suggesting that the membrane surface charge in the immediate vicinity of the membrane may regulate the activity of membrane bound enzymes with charged substrates (Wojtczak and Nalecz, 1979). In this regard, trypsin and phospholipase C may also alter membrane enzyme activities through changes in the surface charge in addition to their direct effect on enzyme activity and their more general effect on the membrane structure itself.

Physiological considerations and conclusions

In conclusion, comparison of the two sarcolemmal preparations used in this study has provided information with respect to the role of the glycocalyx and the influence of membrane sidedness on the properties manifested by this membrane system. With regard to enzymatic properties it would appear both preparations possess qualitative similarities in type of ATPase

activities found in the sarcolemma. Loss of the glycocalyx in sucrose density gradient membranes may have exerted profound effects on the activities measured in this preparation. The orientation of Method I membranes in a predominantly right side out conformation and Method II membranes in a predominantly inside out conformation may also have been responsible for quantitative differences in the enzymatic activities observed. Differences in ouabain sensitivity can be accounted for by the orientation dissimilarity of the preparations but loss of the ouabain binding site accompanying loss of the glycocalyx in Method II membranes cannot be discounted.

The calcium binding characteristics of the two preparations provide an interesting insight into calcium handling by both the cytoplasmic side and the extracytoplasmic side of the rat heart sarcolemma. The preparation of McNamara and associates (1974) displays the well documented extracellular calcium pool which is necessary for excitation-contraction coupling in heart cells. Non-specific binding of calcium, as displayed by this preparation, would seem to require the presence of an intact sarcolemmal membrane complex. The observation that the presence of magnesium strongly inhibited this binding is consistent with the conclusion that calcium bound at this location represents the contractile dependent calcium supply.

The sucrose density gradient preparation, essentially based on the procedure of Kidwai and associates (1971) on the other hand, was shown to lack the intact sarcolemmal membrane complex

and therefore did not possess calcium binding characteristics which would be expected of the extracellular calcium pool, namely inhibition by magnesium at high concentrations. This preparation however, did show ATP dependent calcium binding believed to represent the "calcium pump" responsible for translocating calcium across the sarcolemma to the extracellular compartment. Evidence provided by the cytochemical study of this preparation indicated its inside out orientation and therefore manifestation of characteristics of the calcium pump would be expected.

A close relationship between structure and function is also indicated by the changes seen in several of the cytochemical staining patterns as well as biochemical properties of the membrane following selective extraction or modification of proteins, glycoproteins or phospolipids. Furthermore, these experiments also suggest that any pathological condition influencing these structural components of the membrane would also modify membrane physiology and hence cell function.

Taken in totality this investigation has confirmed the presence of both structural and functional assymetry in the rat heart sarcolemma. Furthermore, it has reinforced the belief that structure and function are interdependent in heart cell membranes and alterations in either of the two, by any means, will be reflected by alteration in myocardial function.

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