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

A Thesis

Presented to the

University of Manitoba

In Partial Fulfilment of the Requirements

for the Degree of

Master of Science in Physiology

by

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April, 1980

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MAST R OF SCIENCE

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Naranjan S. Dhalla for allowing me to pursue my scholastic career and research under his auspices and for his support and interest during the course of this investigation.

I would also like to extend a special word of thanks to Dr. P.K. Singal for his invaluable guidance and friendship.

I am deeply grateful for the love, support and patience of my husband, Ken and my daughter, Deborah Anne, without which none of this would have been possible.

I would also like to express my appreciation to Dr. P.I. Polimeni and to Dr. P. Choy for their helpful suggestions in the preparation of this thesis.

I offer my thanks to my friends and colleagues in the Department of Physiology for their assistance and encourgement.

This study was supported by the Medical Research Council of Canada through a grant extended to Dr. N.S. Dhalla and a studentship awarded to the investigator.

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.