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THE STRUCTURE AND FUNCTION OF THE HEART CELL MEMBRANE: CHARACTERIZATION OF A 360 kDa PROTEIN AND DYSTROPHIN FROM THE MYOCARDIUM

By

Vita Peri

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Dedicated to my parents,

Gregorio and Maria Peri

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ABSTRACT

The Structure and Function of the Heart Cell Membrane: Characterization of a 360 kDa Protein and Dystrophin from the Myocardium

Understanding the molecular architecture of the plasma membrane is of fundamental importance to elucidating the molecular physiology of the myocardium. The voltage-sensitive Ca^{2+} channel, also referred to as the dihydropyridine receptor, plays a key role in excitation-contraction-coupling in cardiac cells. In order to understand the role of the dihydropyridine receptor in excitation-contraction coupling, a thorough study of the structure and function of this molecule is warranted. During the purification of the dihydropyridine receptor on lectin-affinity columns and sucrose density gradients, a high molecular weight protein (360 kDa) was found to co-purify with the receptor. Since several high molecular weight proteins such as ion channels and dystrophin are associated with the cell membrane, the relationship of the 360 kDa protein to these proteins was investigated.

The relationship of the 360 kDa protein with other high molecular weight proteins such as dystrophin was investigated using anti-peptide antibodies against dystrophin. Anti-dystrophin antibodies did not crossreact with the 360 kDa protein and there was no dystrophin detected in the fraction containing the 360 kDa protein. Antibodies that recognized the 360 kDa protein did not crossreact with dystrophin, althcugh subcellular fractionation studies indicated that both dystrophin and the 360 kDa protein were distributed in the cytosol and associated with sarcolemmal membranes. Immunofluorescence studies

conducted in ventricular cardiomyocytes and tissue sections indicated that the 360 kDa protein was localized to the interior of the cell near the cell membrane and throughout the cytosol. Further localization studies conducted in cardiomyocytes using anti-dystrophin antibodies revealed a distinct punctate staining pattern for dystrophin, approximating to the level of the transverse-tubule/Z-line. Lectin staining of purified 360 kDa protein indicated that it was glycosylated. In the presence of a reducing agent such as dithiothreitol, the protein migrated to Mr 180,000 in SDS-gels, suggesting that it exists as a dimer in heart cells. Polyclonal antibodies raised against the purified 360 kDa protein crossreacted with α_2-macroglobulin under both nonreducing and reducing conditions. Furthermore, anti-α_2-macroglobulin antibodies crossreacted with the 360 kDa protein under the same conditions. Following partial proteolysis, both proteins displayed similar digestion products as determined by SDS-PAGE. These results suggested that the 360 kDa protein was related to α_2-macroglobulin.

In view of the function of α_2-macroglobulin as a protease inhibitor, it is speculated that the 360 kDa protein may be performing a similar function in heart cells. The rise in Ca^{2+} during systole may lead to activation of Ca^{2+}-sensitive proteases and the presence of a protease inhibitor in these cells may help prevent the proteolytic degradation of essential proteins such as ion channels and dystrophin. Increased proteolytic degradation in Duchenne muscular dystrophy has been shown to be due to high intracellular Ca^{2+} levels as a result of sarcolemmal membrane damage. The cardiac specific 360 kDa protein may therefore be protecting the dystrophic myocardium from excessive muscle wasting observed in dystrophic skeletal muscle.

ABBREVIATIONS

α_2M	α_2-Macroglobulin
ACh	Acetylcholine
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bis	N,N'-methylene-bis-acrylamide
BLOTTO	Bovine lacto transfer technique optimizer. Buffer containing 5% Carnation powdered milk and 0.02% NaN_2
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
Con A	Concanavalin A
DEAE	Diethylaminoethyl cellulose
DHP	Dihydropyridine
DTT	Dithiothreitol
FITC-IgG	Goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate conjugate
GTP	Guanosine triphosphate
G_i	G inhibitory
G_s	G stimulatory
hr	hour
kDa	kilodalton
MDM	Methyl α-D-mannopyranoside

min	minutes
Mr	Molecular mass
NAG	N-acetyl-D-glucosamine
NBT	Nitro blue tetrazolium
NP-40	NONIDET P-40 (octyl phenol ethylene oxide condensate containing ethylene oxide)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulphonyl fluoride
cRNA	complementary ribonucleic acid
RT	Room temperature
s	seconds
SDS	Sodium dodecyl sulphate
SL	Sarcolemma/Sarcolemmal
SR	Sarcoplasmic reticulum
TBS	Tris buffered saline
TBST	Tris buffered saline - Tween
TEMED	N,N,N',N'-tetramethylethylenediamine
TTX	Tetrodotoxin
T-tubule	Transverse tubule
Tris	Tris[hydroxymethyl]aminomethane
WGA	Wheat germ agglutinin

TABLE OF CONTENTS

LIST OF FIGURES

1.0 INTRODUCTION

The normal physiological function of the heart cell is determined by the proteins that are present in the cell. While a great deal of information has recently been obtained about the molecular physiology of the major proteins, the structure and function of many proteins of the cell membrane still remain to be investigated.

1.1 Cells of the Myocardium

Cardiac muscle cells or myocytes are the characteristic cell type of the heart. These functionally specialized muscle cells are connected end-to-end by specialized complexes called the intercalated discs. These structures link myocytes mechanically, metabolically and electrically in the intact heart, contributing to the excitation-contraction-relaxation cycle of the myocardium.

Myocytes are classified as either working or conductive. Working myocardial cells, ventricular or atrial, are cells highly specialized in the function of contraction or in contraction and secretion, respectively. Conductive cells are those involved in the impulse generation and in the atrio-ventricular conduction system. These cells include the nodal cells of the sino-atrial (SA) and atrio-ventricular (AV) nodes, and the Purkinje cells. The SA node is responsible for the pacemaker activity of the heart and contains myocytes that have special electrophysiological properties enabling them to generate spontaneously the electrical impulse responsible for heart contraction. The AV node slows the conduction impulse and enables sequential contraction of atria and ventricles (atrio-ventricular impulse conduction). It in turn gives rise to pathways which penetrate into the ventricles and ramify to form the Purkinje fiber system. The Purkinje fibers effect rapid conduction of the electrical impulse throughout the heart.

1.2 Ultrastructure of the Myocardium

At the microscopic level, working cells can be clearly distinguished from conductive cells. Both cell types contain myofibrils, mitochondria, various membrane systems, cytosolic components and nuclei. However, conductive cells because they do not actively contract, contain fewer mitochondria and myofibrils than the working cells and are composed mainly of cytoplasm. The myofibrils are an important constituent of the working cell next to the mitochondria and membrane systems. Occupying about half of the cell volume, the myofibrils are composed of thick (myosin) and thin (actin) filaments which give the characteristic striated appearance. These structures associate with regulatory contractile and cytoskeletal components in order to achieve muscle contraction. The sarcomere is the basic contractile unit of striated muscle. Bounded by the Z-line, the sarcomere contains two half I-bands and one A-band (see Fig. 1). The region of the sarcomere occupied by the thick filaments into which thin filaments extend from either side is known as the A-band. The I-band is the region of the sarcomere occupied by only thin filaments. These extend toward the centre of the sarcomere from the Z-lines.

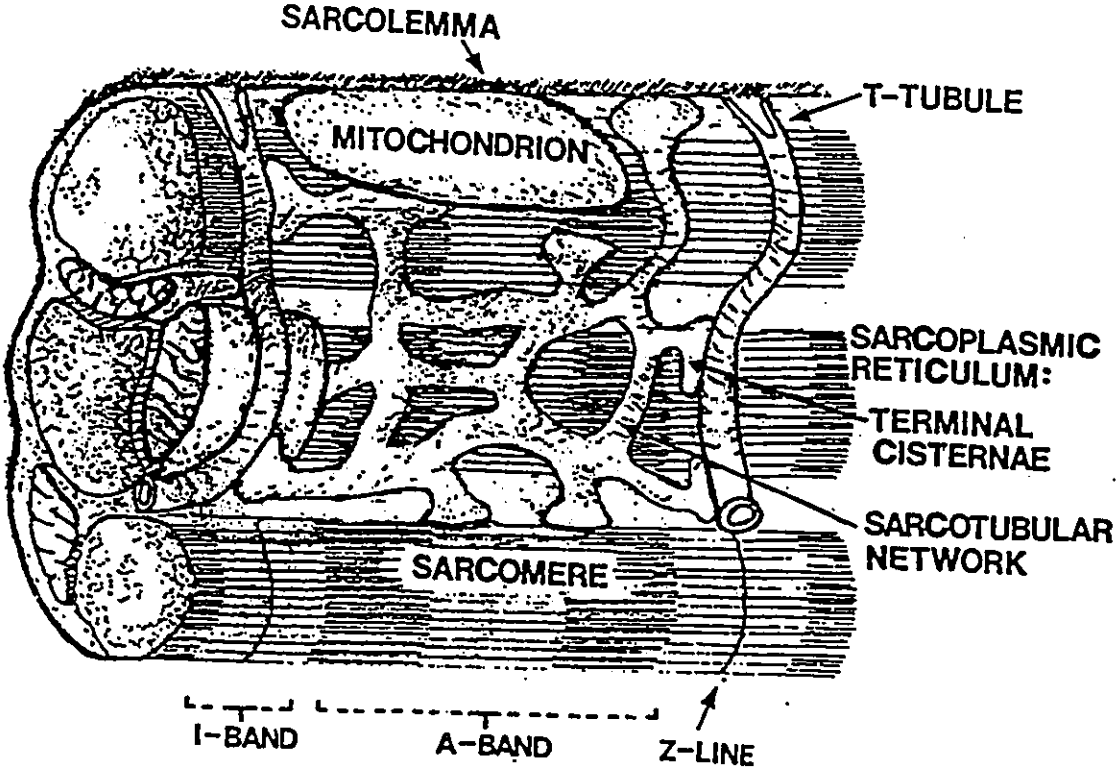
1.3 Membrane Systems and Excitation-Contraction-Coupling

The various membrane systems found within cardiac muscle cells are vital in maintaining normal cardiac function. The sarcolemma (SL), transverse tubules (t-tubules), sarcoplasmic reticulum (SR) and mitochondria are intimately involved in signal transduction and energy metabolism as well as regulation of ion movements during the excitation-contraction-relaxation cycle of the myocardium.

Cardiac muscle contraction is initiated by the depolarization of the SL membrane (Adams, 1980). Action potential generation is mediated by voltage-

Figure 1: Schematic diagram of the salient structures involved in excitation-contraction coupling:

The contractile proteins are arranged in a regular array of thick and thin filaments. The A-band represents the region of the sarcomere occupied by the thick filaments into which thin filaments extend from either side. The I-band is the region of the sarcomere occupied only by thin filaments extending toward the centre of the sarcomere from the Z-lines. The sarcomere, the basic contractile unit, is the region between each pair of Z-lines and contains two half I-bands and one A-band. The sarcolemma, which surrounds the cell, invaginates at regular intervals forming the t-tubular system. The region where the t-tubules abut the sarcoplasmic reticulum is referred to as the terminal cisternae. The sarcoplasmic reticulum, which surrounds the myofibrils, consists of a network of tubules called the sarcotubular network. Mitochondria are visible throughout the cell providing the energy for contraction. Figure adapted from (Katz, 1977).


SARCOLEMMA
T-TUBULE
MITOCHONDRION
SARCOPLASMIC RETICULUM:
TERMINAL CISTERNAE
SARCOTUBULAR NETWORK
SARCOMERE
I-BAND
A-BAND
Z-LINE

gated Na^+ channels in the SL resulting in the influx of Na^+ and depolarization of the cell. This depolarization results in the activation of slow voltage-gated Ca^{2+} channels, allowing extracellular Ca^{2+} to enter the cell down its concentration gradient. This gives the characteristic plateau phase of the cardiac action potential. Extracellular Ca^{2+} however is not enough to activate the contractile proteins to cause muscle contraction. Evidence indicates that the influx of extracellular Ca^{2+} induces a larger release of Ca^{2+} from the SR (Fabiato, 1977). This net increase in cytosolic Ca^{2+} results in the interaction between the contractile proteins leading to contraction of the myocardium. As the action potential continues, Ca^{2+} channels begin to spontaneously inactivate and the SL becomes progressively impermeable to Ca^{2+}. Through Ca^{2+} pumps located within the SR and SL and the Na^+/Ca^{2+} exchanger located in the SL, Ca^{2+} is removed from the cytosol resulting in relaxation of the myocardium. Finally, voltage-gated K^+ channels open resulting in an outward K^+ current, ending the action potential and restoring the membrane potential.

1.3.1 The Mitochondria:

The principal function of the mitochondria is to generate ATP. Located adjacent to the myofibrils and beneath the SL, mitochondrial membranes transduce energy which is made available when ions move along their respective electrochemical gradients resulting in the synthesis of ATP. This energy is then utilized for muscle contraction as well as for various metabolic processes. Mitochondrial oxidative phosphorylation provides about 90% of the ATP utilized by the working heart (Crompton, 1990). The mitochondria has the ability to accumulate large amounts of Ca^{2+}, acting as a sink for intracellular Ca^{2+} (Carafoli, 1971; Carafoli, 1985). However, the observed mitochondrial Ca^{2+} fluxes were shown to be too slow to be taking part in regulating Ca^{2+}

during the contraction-relaxation cycle (Bers, 1989). The observed Ca^{2+} fluxes are believed to regulate intramitochondrial processes. The storage of large amounts of Ca^{2+} by the mitochondria in situations of severe cellular Ca^{2+} overload, particularly under pathological conditions such as ischemia (Reimer, 1986), may serve as an important safety device for heart cells by protecting the cytoplasm from very high Ca^{2+} levels.

1.3.2 The Sarcoplasmic Reticulum:

The SR is the major intracellular membrane system of muscle cells, consisting of a plexiform arrangement of tubular elements surrounding each myofibril (see Fig. 1). It is the primary storage site for Ca^{2+} within the cell. The SR functions mainly in the release of Ca^{2+} to and sequestration of Ca^{2+} from the myoplasm thus achieving contraction and relaxation, respectively. Relaxation is brought about in part by the active Ca^{2+} pump, the Ca^{2+} -Mg^{2+} ATPase, a protein of Mr 110,000. The Ca^{2+} -Mg^{2+} ATPase is stimulated by micromolar concentrations of Ca^{2+} and utilizes Mg^{2+} and ATP as substrates. Two Ca^{2+} ions and one ATP molecule bind to high affinity binding sites on the cytoplasmic side of the pump. The pump is regulated by the phosphorylation of the protein phospholamban, a homopentamer of Mr 22,000 (Tada, 1982). Phosphorylation has been reported to be achieved by cAMP-dependent protein kinase (Tada, 1974) as well as by calmodulin-dependent protein kinase (Kirchberger, 1982; Simmerman, 1986). The transported Ca^{2+} is then sequestered and stored primarily by the Ca^{2+} binding protein calsequestrin (Carafoli, 1985), originally described in skeletal muscle by MacLennan (MacLennan, 1971). Campbell *et al* (1983) later identified and purified calsequestrin from cardiac muscle. The cardiac isoform of calsequestrin has a Mr 45,000 as determined by cDNA cloning, however migrates in

SDS-gels with a Mr of 55,000. Calsequestrin is localized in the terminal cisternae, where it is associated as a matrix with the luminal surface junctional face membrane (Jorgensen, 1977).

The SR forms functionally specialized structures where it comes in contact with the SL and t-tubules (see Fig. 1). Here, the tubules of the SR flatten to form the junctional SR or terminal cisternae. One SR cisternae abuts to one t-tubule in cardiac muscle and is termed the diad. The membrane-protein composition and morphology of the junctional SR appears to be different from the free SR. Bridging structures or spanning proteins called "feet" (Franzini-Armstrong, 1970) are seen at the junction of the SR with either the SL or t-tubules. Ultrastructural studies have shown the existence of clusters of diamond shaped electron dense structures originating in the junctional face membrane of the SR and crossing the 150 A° gap to touch the t-tubular membrane. Based on their distinctive morphology and high affinity for the neutral plant alkyloid ryanodine, these feet structures have recently been purified and identified as the Ca^{2+}-release channel/ryanodine receptor (Inui, 1987; Lai, 1988). The channel is a homotetramer, each monomer of Mr 560,000. It can be activated by caffeine (Rousseau, 1988), ATP (Rousseau, 1989) and Ca^{2+} and inactivated by Mg^{2+} (Rousseau, 1988) and micromolar concentrations of calmodulin (Meissner, 1987). Block *et al* (1988) demonstrated in skeletal muscle that the feet are organized in a distinct pattern on the SR underneath the t-tubular membrane and are matched by an organized array of particles in the t-tubular membrane, the sarcolemmal Ca^{2+} channel protein/dihydropyridine (DHP) receptor. Four DHP receptors match every other ryanodine receptor. This model allows for the direct communication of the DHP receptor with the Ca^{2+} release channel and is consistent with the

calcium-induced-calcium-release hypothesis described in cardiac muscle by Fabiato & Fabiato (1979).

1.3.3 The Sarcolemma and Transverse Tubules:

The SL is a dynamic membrane system which surrounds the myocardial cell, defining the boundary between the intracellular and extracellular spaces (see Fig. 1). It consists of an outer and an inner layer. The outer layer, termed the glycocalyx is composed of mucoplysaccharides, glycoproteins and sialic acid residues. This chemical composition contributes to the fixed negative charge in this region and has a high capacity for cation binding, particularly Ca^{2+} (Adams, 1980). The glycocalyx may be a site of Ca^{2+} binding and exchange across the cell membrane and may play a role in excitation-contraction coupling. The inner layer, termed the plasma membrane, consists of a lipid bilayer (Langer, 1982). Embedded within the lipid bilayer are various proteins and glycoproteins comprising ion channels, signal transducing components and regulatory components. At regular intervals, the SL invaginates to the cell interior forming the t-tubular system. In cardiac muscle, the t-tubules invaginate predominately at the Z-line of the sarcomere. The t-tubules function in the transmission of the action potential down into the interior of the cell and in controlling the flux of Ca^{2+} into the myoplasm.

One of the main functions of the SL is to induce and maintain an electrochemical gradient of various ions, particularly Na^{+}, Ca^{2+} and K^{+}, which play an essential role in cardiac contractility and relaxation. Some proteins embedded within the SL form ion channels and ion carriers as well as enzymes which allow ions to be transported across the membrane and signals to be transduced. Various other proteins act as receptor sites for hormones and drugs modulating ion fluxes (hence contractility) and various cellular activities

(metabolism). Other proteins still, which are believed to take part in cardiac homeostasis, remain to be identified.

1.4 Ion Carriers and Transport Enzymes

Various ion carriers and enzymes have been identified in the SL. Among these, the Na^+-K^+ ATPase, the Na^+-Ca^{2+} exchanger and Ca^{2+} pump have been shown to be most important in restoring and maintaining intracellular ionic concentrations particularly of Ca^{2+}, during the excitation-contraction-relaxation cycle of the myocardium.

1.4.1 The Na^+-K^+ ATPase:

During depolarization, small amounts of Na^+ enters the cell along the Na^+ gradient. In order for the cell to remain excitable, it is essential that the Na^+ gradient be restored. This is achieved by the Na^+-K^+ ATPase or Na^+ pump. The pump is electrogenic transporting three Na^+ out of the cell in exchange for two K^+ in for every ATP hydrolyzed (Glitsch, 1982). The protein consists of two α and two ß subunits linked together noncovalently. The α subunit with a Mr 100,000, is a large membrane spanning protein with a cytoplasmic domain where ATP binding and phosphorylation take place (Jorgensen, 1988). The ß subunit is glycosylated having a Mr 40,000. Its function is not clear yet, although recent evidence indicates that it facilitates the correct assembly and transport of the α subunit into the plasma membrane (Geering, 1990).

1.4.2 The Na^+-Ca^{2+} Exchanger:

The Na^+-Ca^{2+} exchanger is an important mechanism reponsible for the beat to beat efflux of Ca^{2+} from the myocardium. In studies using SL vesicles, it was determined that three Na^+ are exhanged for each Ca^{2+}, making it

electrogenic (Pitts, 1979). Several studies have demonstrated that the Na^+-Ca^{2+} exchanger is sensitive to transmembrane potential and is also capable of generating a voltage gradient (Caroni, 1980a; Philipson, 1980; Reeves, 1980). The molecular properties of the exchanger have been recently described. Philipson *et al* (1988), correlated the cardiac SL Na^+-Ca^{2+} exchanger activity with proteins of Mr 70,000 , 120,000 and 160,000. The 70 kDa protein is believed to be an active proteolytic fragment of the 120 kDa protein, although differential processing may also account for the multiple protein bands. The exchanger has also been shown to be glycosylated. The cDNA encoding the cardiac Na^+-Ca^{2+} exchanger has recently been cloned (Nicoll, 1990) and the predicted primary structure indicates twelve membrane-spanning segments and a large cytoplasmic domain which contains a calmodulin binding site and a potential phosphorylation site which could be a substrate for calmodulin or cAMP-dependent protein kinase. The intracellular surface of the exchanger has a regulatory Ca^{2+} binding site that modulates the Na^+-Ca^{2+} exchanger activity (Kimura, 1986). ATP stimulation of the Na^+-Ca^{2+} exchanger in SL vesicles (Caroni, 1983) and in giant excised SL membrane patches (Hilgemann, 1990) has been observed. It is believed however that ATP-dependent stimulation is due to regulatory phosphorylation of the exchanger rather that an allosteric effect of ATP or use of ATP as an energy source (Bers, 1991).

1.4.3 The Ca^{2+} Pump:

The cardiac sarcolemmal Ca^{2+} pump or Ca^{2+} ATPase was first described in SL vesicle studies (Caroni, 1980b). Identified as a protein of Mr 138,000 (Niggli, 1981; Verma, 1988), it extrudes one Ca^{2+} per ATP hydrolyzed. The Ca^{2+} pump is stimulated by cAMP dependent phosphorylation and by

calmodulin (Caroni, 1981a; Caroni, 1981b; Tuana, 1981). The pump has a high affinity for Ca^{2+} and its rate of Ca^{2+} extrusion is too slow for it to play a role in relaxation of the myocardium. The high affinity for Ca^{2+} suggests that the pump operates continuously, extruding Ca^{2+} from the cell during both diastole and systole. By contrast, the Na^{+}-Ca^{2+} exchanger with a much lower affinity for Ca^{2+}, operates efficiently when intracellular Ca^{2+} has increased significantly. It is believed that the Ca^{2+} pump is more important in the slower, longer term extrusion of Ca^{2+} by the cell, acting as a fine tuner of cell Ca^{2+} responsible for maintaining Ca^{2+} at the submicromolar concentrations. This avoids intracellular Ca^{2+} overload and cell damage.

1.5 Receptors and Second Messenger Systems

Many sarcolemmal receptors involved in signal transduction have been identified and characterized. Among these are the ∝- and ß-adrenoceptors, cholinergic, dopaminergic, serotoninergic, histaminergic and angiotensin II receptors. Receptors are acted on by a variety of endogenous and exogenous agents having both stimulatory and inhibitory effects on cardiac function. These include neurotransmitters (norepinephrine, epinephrine and acetylcholine), hormones (histamine and angiotensin II) and a variety of drugs (ß-blockers and cardiac glycosides). Once stimulated, receptors exert their actions via the activation of second messenger systems involving proteins such as adenylate cyclase, G proteins and cAMP.

1.5.1 ß-Adrenerginc Receptors:

Two ß-receptor types have been identified, termed $ß_1$ and $ß_2$. These receptors are glycoproteins and vary in molecular masses and abundance among species and locations within the body. Mammalian cardiac ventricles

contain predominately the β_1 type (Stiles, 1984). The β_1 receptors are primarily involved in inotropic cardiac responses, whereas the β_2 are involved in chronotropic responses. Agonists for ß-adrenergic receptors have been shown to increase Ca^{2+} current into the cell (Reuter, 1967). It is now known that occupation of the ß-adrenergic receptor by an agonist leads to the activation of a GTP binding protein (G protein) known as G_s which, in turn, activates adenylate cyclase to produce cAMP. The increase in cAMP leads to the dissociation of the regulatory and catalytic subunits of the cAMP-dependent protein kinase. The catalytic subunit of cAMP-dependent protein kinase phosphorylates several proteins including the L-type Ca^{2+} channel. This phosphorylation leads to activation of the channel, causing an increase in Ca^{2+} currents. Recently, it has been shown that activation of G_s by ß-adrenergic agonists can exert a direct effect to increase channel activity, bypassing the slower, cAMP-dependent pathway (Yatani, 1987; Yatani, 1989).

1.5.2 Cholinergic Receptors:

Cholinergic receptors are classified as nicotinic or muscarinic, with the muscarinic type being subdivided into two subtypes, M_1 and M_2. The M_2 subtype (Mr 80,000), appears to be enriched in plasma membrane preparations. In the early 1980's, evidence suggested that acetylcholine (ACh) had an inhibitory effect on Ca^{2+} currents in cardiac tissue (Fischmeister, 1986). It is now known that this inhibition is due to an ACh induced and G-protein-mediated (G_i) inhibition of adenylate cyclase and therefore a decreased cAMP level. This inhibitory effect was observed to be much more prominent when Ca^{2+} current was enhanced via ß-adrenergic agonists or cAMP. ACh also stimulates guanylate cyclase resulting in elevated cGMP. cGMP promotes

cAMP hydrolysis via stimulation of a cyclic nucleotide phosphodiesterase, thereby reducing cAMP and Ca^{2+} currents (Watanabe, 1975; Flitney, 1981).

1.5.3 Other Receptors:

Histamine and angiotensin II have both been shown to alter Ca^{2+} currents in cardiac muscle by binding to their receptors, H_2 and angiotensin II, respectively. Histamine activates adenylate cyclase and the cAMP cascade in much the same manner as ß-receptor agonists, leading to an increase in Ca^{2+} currents (Levi, 1988). Angiotensin II also increase Ca^{2+} currents, but apparently via stimulation of protein kinase C rather that cAMP-dependent protein kinase (Allen, 1988).

1.6 Ion Channels

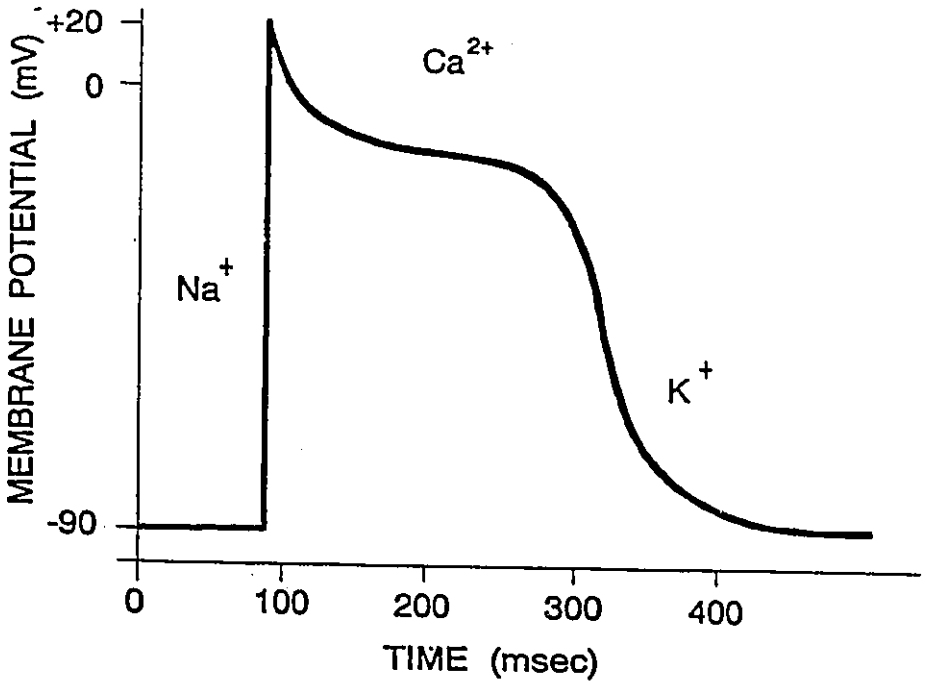
There are several ion channels found within the SL. Of particular interest are the voltage-sensitive ion channels which are involved in the generation and maintenance of the action potential in excitable cells (see Fig. 2A). These channels mediate rapid, voltage-gated changes in ion permeability (Catterall, 1988). In the heart, three voltage-sensitive ion channels are intimately involved in the formation of the action potential. These include the Na^+, K^+ and Ca^{2+} channels. These voltage-sensitive channels have been shown to consist of a principal transmembrane subunit which forms the ion conducting pore and is associated with other subunit(s) believed to perform regulatory functions. Based on cDNA cloning experiments, transmembrane arrangements of the principal subunits of the channels have been proposed (see Fig. 2B). The channels contain six membrane spanning segments designated S_1 through S_6. The K^+ channel contains one S_1-S_6 domain, whereas both the Na^+ and Ca^{2+} channels contain four of these domains. This suggests an evolution of these channels

Figure 2: The cardiac action potential and the voltage-sensitive ion channels:

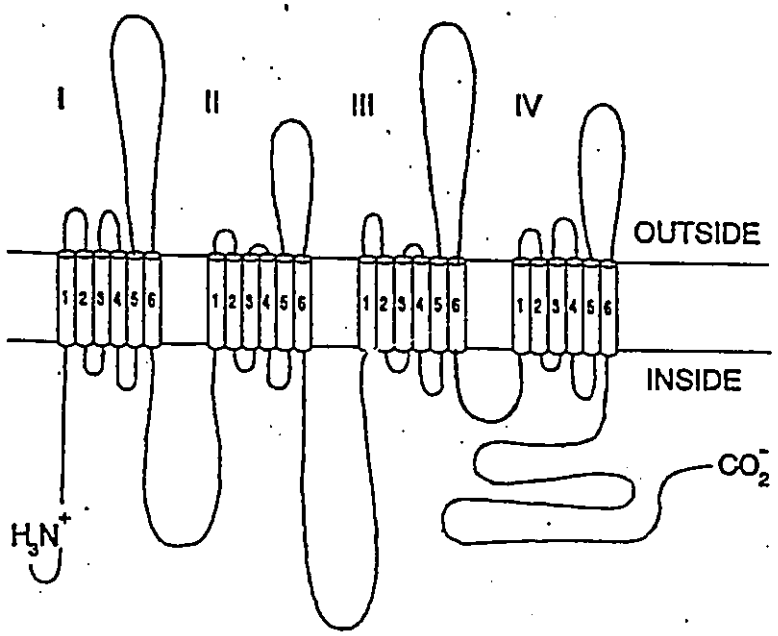
(A) The cardiac action potential (AP) lasts just under 400 msec. The Na^+, Ca^{2+} and K^+ voltage-sensitive ion channels are involved in shaping the action potential. The upstroke of the AP is due to the influx of Na^+ mediated by the voltage-sensitive Na^+ channel. This is followed by the influx of Ca^{2+} through the "slow" L-type voltage-sensitive Ca^{2+} channel. The SL membrane is then repolarized by the outward movement of K^+ through the voltage-sensitive K^+ channel, leading to the end of the AP.

(B) Schematic of the predicted topology of the α_1 subunit of the DHP receptor. The Na^+ and Ca^{2+} channels' α and α_1 subunits, respectively, are composed of four homologous domains, I-IV, each having six transmembrane α-helix segments, represented by the S_1-S_6 cylinders. The K^+ channel however, contains only one domain of six transmembrane segments. The S_4 segment is the most conserved structure of the voltage-sensitive channels and functions as a voltage sensor. Figure is adapted from (Catterall, 1988).

A


MEMBRANE POTENTIAL (mV)
+20
0
-90
Ca2+
Na+
K+
0
100
200
300
400
TIME (msec)

B


I
II
III
IV
OUTSIDE
INSIDE
1 2 3 4 5 6
CO2-
H3N+

from a single gene. The amino acid sequence is very similar among the channels and is conserved in the proposed transmembrane regions. The S_4 segment present in each domain is highly conserved among the channels and contains a stretch of positively charged amino acid residues proposed to serve as a voltage sensor (Catterall, 1988; Tuana, 1990). These channels are also glycosylated, yet the role of this glycosylation is not known.

1.6.1 The Na^+ Channel:

During the initial phase of the action potential, a rapid increase in Na^+ permeability causes a rapid depolarization of the SL. This is mediated by voltage-sensitive Na^+ channels. Following depolarization, there is a rapid closure of the channels preventing Na^+ from entering the cell. Voltage-sensitive Na^+ channels in mammalian heart differ from those in nerve and skeletal muscle. One major difference is that tetrodotoxin (TTX)-resistant cardiac Na^+ channels are blocked by 1-10 μM TTX, whereas TTX-sensitive nerve Na^+ channels are blocked by nanomolar TTX concentrations (Fozzard, 1985). The TTX-resistant cardiac-specific channel mRNAs appear also upon denervation of skeletal muscle (Rogart, 1989; Yang, 1991). The Na^+ channel was first isolated and sequenced from eel electroplax (Noda, 1984) and shown to be composed of a single subunit of Mr 260,000. The Na^+ channel has also been purified from other tissues and shown to contain different subunit compositions. The Na^+ channel from mammalian brain consists of three subunits, an α, a β_1 and a β_2 with Mr of 260,000, 36,000 and 33,000, respectively (Hartshorne, 1984). The β_2 subunit is attached to the α subunit by disulfide bonds. All three subunits are heavily glycosylated. The Na^+ channel isolated from skeletal muscle contains only two subunits, the α and the β_1 with Mr 260,000 and 38,000, respectively (Kraner, 1985; Casadei, 1986). The α subunit from rat cerebral cortex, rat

heart and eel electroplax but not from skeletal muscle, was shown to be phosphorylated by cAMP dependent protein kinase (Gordon, 1988).

1.6.2 The K^+ Channel:

Termination of the action potential is achieved by activation of voltage-sensitive K^+ channels. These channels mediate the outward movement of K^+ ions which repolarizes the cell. The K^+ channels are also involved in setting the resting membrane potential and modulating the action potential frequency and threshold. Several types of voltage-sensitive K^+ channels exist. One type is the delayed rectifier K^+ channel which is found in most nerve and muscle cells. It is activated slowly following membrane depolarization and primarily acts to repolarize the action potential. Another type is the transient A-channel, which rapidly activates and inactivates and is important for shaping the action potential (Connor, 1971). These channels are found in neurons of the central and peripheral nervous system (Rudy, 1988). Several other voltage-sensitive K^+ channels exist having intermediate properties (Rudy, 1988). The *Shaker* locus of *Drosophila* has been shown to encode a family of A channels which are expressed in neurons and muscle (Tempel, 1987; Timpe, 1988). The proteins have a Mr 70,000 and have homology with both the Na^+ and Ca^{2+} channel segment regions of these proteins (McKinnon, 1989). Several structural similarities have been found between A channels and delayed rectifier channels. Moreover, the sequence of the S_4 segments, which renders an ion channel voltage sensitive, have been shown to be almost identical among the various K^+ channels (Gordon, 1990).

1.6.3 The Ca^{2+} Channel:

The Ca^{2+} channel plays an important role in achieving contraction of the myocardium. Following the inactivation of the Na^{+} channel, the cardiac action potential shows a slow inward current of Ca^{2+}, giving the characteristic plateau shape of the action potential in cardiac muscle. It is the L-type voltage-sensitive Ca^{2+} channel which mediates the movement of Ca^{2+} from the extracellular space to the interior of the cell. This Ca^{2+} movement is an obligatory step in the initiation of myocardial contraction.

As this laboratory has been involved in researching the L-type voltage-sensitive Ca^{2+} channel from both cardiac and skeletal muscle and forms the basis of this thesis, the remainder of this introduction will focus on the Ca^{2+} channel.

1.7 The Voltage-Sensitive Ca^{2+} Channels

The co-existence of multiple types of voltage-sensitive Ca^{2+} channels within a given type of cell was first described in voltage-clamp studies on starfish eggs (Hagiwara, 1975). Subsequent voltage-clamp and patch-clamp experiments in various cells demonstrated the presence of three kinds of voltage-sensitive Ca^{2+} channels, the L-type or "long-lasting", T-type or "transient" and N-type or "neuronal". The N-type is found solely in neuronal tissue, whereas L- and T-types are found in a wide variety of neuronal, endocrine, exocrine and muscle cells. Both the L- and T-type Ca^{2+} channels appear in cardiac muscle, however, the T-type appear to be prominent in atrial and Purkinje cells (Hirano, 1989). The functional significance of the T-type channel is not known, though it may be involved in pacemaker activity (Hagiwara, 1988).

The L-type channels are characterized by a large unitary conductance (~25 pS), long lasting openings, activation at larger depolarizations and sensitivity to the 1,4-dihydropyridines (DHPs) Ca^{2+} channel blockers. T-type channels are characterized by a tiny unitary conductance (~8 pS), transient openings, activation at more negative membrane potentials and are insensitive to the DHPs. The N-type channels are intermediate in conductance and voltage dependence when compared with the L- and T-types.

It is the L-type Ca^{2+} channel which is responsible for the majority of Ca^{2+} entry in ventricular muscle. The sensitivity of the channel to the DHPs has allowed these drugs to be used as molecular probes to identify the channel components.

1.8 Ca^{2+} Channel Blockers

The Ca^{2+} channel blockers or Ca^{2+} channel antagonists are a heterogeneous group of compounds that can be divided into three structurally distinct classes (Scriabine, 1987). They consist of the 1,4-dihydropyridines which are exemplified by nifedipine, the phenylalkylamines exemplified by verapamil and the benzothiazepines exemplified by diltiazem (Fleckenstein, 1977). Electrophysiological studies indicated that these compounds specifically inhibit the transmembranal flow of Ca^{2+} through the "slow", L-type voltage-sensitive Ca^{2+} channel (Reuter, 1983). Due to their ability to inhibit the flow of Ca^{2+} across the cell membrane thereby modifying the electrical and contractile properties of the muscle, these blockers are used clinically in the treatment of various cardiovascular disorders such as angina, cardiac arrythmias and hypertension (Godfraind, 1986).

The development of tritiated analogues of the various Ca^{2+} channel blockers has allowed for the identification of binding sites for these compounds

in cell membrane preparations. Various binding studies have indicated that there are three distinct binding sites on the Ca^{2+} channel, one for each class of blocker, which are allosterically linked (Glossmann, 1985; Triggle, 1987; Tuana, 1987; Murphy, 1988). However, the DHPs have been shown to have the highest affinity for the channel and the slowest rate of dissociation (Janis, 1984; Glossmann, 1985). The high affinity of these compounds for their binding sites allowed the initiation of biochemical studies aimed at identifying the structure of the Ca^{2+} channel.

1.9 Biochemical Characterization of the L-Type Ca^{2+} Channel

The isolation and complete biochemical characterization of the subunits of the L-type Ca^{2+} channel have been achieved only from rabbit and guinea pig skeletal muscle t-tubule membranes. These membranes are the richest source of Ca^{2+}-antagonist receptor sites known (Ferry, 1982). The density of Ca^{2+}-antagonist receptor sites in mammalian brain, cardiac or smooth muscle membranes is 20 to 500 times lower than in skeletal muscle (Janis, 1987) making purification from these tissues extremely difficult. Additionally, the stability against protease attack is also a more serious problem.

The first report of the purification of the DHP receptor was from skeletal muscle t-tubules (Curtis, 1984). Purification was accomplished by firstly solubilizing the drug labelled membranes with the detergent digitonin and following the labelled proteins through a combination of lectin affinity chromatography, ion exchange chromatography and centrifugation through a sucrose density gradient. The final purified preparation appeared to contain two polypeptides. One large polypeptide termed the α subunit, appeared as a single band under nonreducing conditions in SDS-gels having a Mr 170,000. However upon reduction with dithiothreitol, the band shifted to Mr 140,000 with

the appearance of an additional band at Mr 30,000, presumably released from the large polypeptide during disulfide group reduction. This was termed the δ subunit. A second polypeptide having a Mr 50,000-55,000 termed the ß subunit, did not shift under reducing conditions. It was not clear which polypeptide contained the binding site for the Ca^{2+}-antagonist or whether these were proteolytic products. The apparent heterogeneity in the region was first explained by the incomplete reduction of the intrachain disulfide bonds (Curtis, 1984) or possibly the presence of contaminating proteins (Flockerzi, 1986).

This controversy was later resolved with the development of arylazido photoactive DHP derivatives which were used in photoaffinity labelling experiments. Several investigators, including this laboratory, found that [^{3}H]azidopine, in the presence of *uv* light, was specifically incorporated into a polypeptide of 170 kDa in skeletal muscle t-tubules (Campbell, 1988; Catterall, 1988b; Tuana, 1988; Vaghy, 1988). The migration of the [^{3}H]azidopine labelled polypeptide in SDS-gels was not altered by reduction (Sharp, 1987) and was therefore not the original subunit previously described. This subunit was termed α_1 and the original termed α_2. Following the use of fresh t-tubular skeletal membrane preparations and the addition of a full complement of protease inhibitors, the composition of the skeletal muscle channel was deduced. The channel is accepted to be composed of 5 subunits, α_1, α_2, ß, γ and δ of Mr 170,000, 140,000 (reduced), 52,000, 30,000 and 28,000, respectively, in a stoichiometry of 1:1:1:1 (α_1:α_2-δ:ß:γ) (Campbell, 1988; Leung, 1988). Further studies have identified three forms of the δ peptide (Jay, 1991). These have been termed δ_1, δ_2 and δ_3 having Mr 25,000, 22,000 and 17,000, respectively, in stoichiometric ratios of 1.0:0.31:0.47:0.08 for α_2:δ_1:δ_2:δ_3. These are believed to arise from post-translational modifications of the α_2 subunit, but their physiological role is unclear.

The isolation of the subunits of the Ca^{2+} channel from cardiac muscle has been hampered by low abundance and proteolytic degradation. Using photoaffinity labelling experiments and antibodies raised against peptides of the skeletal muscle channel subunits, related α_1 and α_2 subunits have been identified and partially purified from the cardiac muscle (Cooper, 1987; Tuana, 1987). The cardiac α_1 subunit appeared to migrate more slowly on SDS-gels having Mr 185,000-190,000 as compared with skeletal muscle. To-date, attempts at completely purifying the subunits of the channel from cardiac muscle have not been successful. The exact subunit composition which is believed to confer some of the functional differences between these two channels, is unknown.

1.10 Functional Characteristics of the L-Type Ca^{2+} Channel

The α_1 subunit bears the main known functional characteristic of the L-type Ca^{2+} channel. Cloning and expression of the cDNA of the α_1 subunit together with reconstitution studies, have revealed that this polypeptide is the key functional protein as it possesses the ability to form a channel and it contains the receptor sites for the various types of drugs that are capable of activating or inhibiting these channels (Mikami, 1989; Perez-Reyes, 1989). When reconstituted in phospholipid vesicles, the purified DHP receptor from skeletal muscle was capable of mediating Ca^{2+} fluxes in a DHP-sensitive manner (Curtis, 1986). Similarly, electrophysiological studies demonstrated that purified receptor incorporated into planar lipid bilayers could generate Ca^{2+} currents in a DHP-sensitive manner (Flockerzi, 1986).

Phosphorylation studies indicated that both the α_1 and ß subunits are substrates for cAMP-dependent- (Curtis, 1985; Tuana, 1988, Chang, 1991) and Ca^{2+}/calmodulin-dependent-protein kinase (Hosey, 1986; Hosey, 1987;

Murphy, 1989) as well as for protein kinase C (Hofmann, 1987; Imagawa, 1987; O'Callahan, 1988; Chang, 1991). Phosphorylation increased the channel activity and this was attributed to an increase in the mean open time and a decrease in the closed time of the reconstituted DHP receptors (Flockerzi, 1986; Chang, 1991). The amino acid sequence of these subunits indicates they contain consensus sequences that could serve as substrates for protein kinases in agreement with biochemical studies (Perez-Reyes, 1989; Ruth, 1989).

Co-expression of the cardiac α_1 subunit cDNA and skeletal α_2 subunit cDNA in *Xenopus* oocytes increased Ca^{2+} fluxes in comparison to the cardiac α_1 subunit alone (Mikami, 1989) Recently, the functional role of the α_2-δ subunits were further assayed. Partially purified holomeric channels (α_1 subunit) and separated α_2-δ subunits were reconstituted into liposomes and their properties measured using an assay based on the Ca^{2+} indicator dye fluo-3 (Gutierrez, 1991). Reconstituted channels that were 85% depleted in α_2-δ subunits showed a significant decrease in the initial rate of influx. When the separated α_2-δ subunits were added back to the subunit-containing preparation, the channels exhibited their normal rate of Ca^{2+} influx. In another study, cRNAs of cardiac α_1 subunit and of skeletal muscle α_2-δ, β and γ subunits were synthesized *in vitro* and injected into *Xenopus* oocytes (Singer, 1991). Co-expression of α_1 with α_2-δ fine tuned the activation kinetics of the cardiac channel expressed in the oocyte. The α_2-δ combined with β increased the voltage sensitivity of activation. Addition of α_2-δ, α_2-δ + β or γ to the α_1 subunit, sharply increased the voltage sensitivity of the inactivation process. The α_2-δ + β but not β, also strongly accelerated the current decay. The effect of the γ subunit was especially significant. It dominated the inactivation process when present, making it faster and more sensitive to voltage. Similar results were obtained with murine L cells cotransfected with various

combinations of expression plasmids for α_2, ß, and γ subunit cDNAs (Varadi, 1991).

Further biochemical studies along with the availability of cDNA clones for the subunits of the DHP receptor from skeletal and cardiac muscle, should allow for the assessment of the precise structure and function of the cardiac Ca^{2+} channel.

1.11 Objectives

The L-type voltage-sensitive Ca^{2+} channel from cardiac muscle SL membranes has been identified and partially purified in this laboratory. The purification has been hampered by low abundance and proteolytic degradation of the receptor. During purification, a major high molecular weight protein (360 kDa) was found to be present in the partially pure preparation. High molecular weight proteins such as the Na^+ channel (Noda, 1984), Ca^{2+} channel (Campbell, 1988) and ryanodine-receptor-Ca^{2+}-release channel (Inui, 1987) and dystrophin (Campbell, 1989) are high molecular weight proteins known to play important roles in muscle function. The 360 kDa protein may therefore be related to these group of molecules. In order to study these possibilities a more efficient and simplified protocol for the purification of the 360 kDa protein was required. The objective of this study was therefore to develop a simplified purification protocol for the 360 kDa protein in order to study its structure and functional significance.

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Tissue Specimens:

Fresh porcine hearts were purchased from Abattoir Brisson Slaughterhouse, (Embrun, Ontario). Fast twitch skeletal muscle was obtained from New Zealand white rabbits from the National Reseach Council (Ottawa, Ontario). Hearts and skeletal muscle from *mdx* mice were a generous gift from Dr. Paul Holland, Neurological Institute, McGill University (Montréal, Québec). Cardiomyocytes from New Zealand white rabbits were obtained from Dr. Michel Désilets, Department of Physiology, University of Ottawa (Ottawa, Ontario).

2.1.2 Chemical Reagents and Compounds:

Acrylamide, bis-acrylamide, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), glycine and other electrophoresis reagents were purchased from Bio-Rad, (Richmond, CA) as well as Coomassie Brilliant Blue R-250 and alkaline phosphatase-conjugate-goat anti-rabbit IgG. Trizma base, SDS-PAGE molecular weight markers, Ponceau S. stain, disodium pyrophosphate, sodium pyrophosphate, *p* - phenylenediamine, peroxidase-linked wheat-germ lectin, peroxidase-linked concanavalin A lectin, digitonin, protease inhibitors, methyl ∝-D-mannopyranoside (MDM), N-acetyl-D-glucosamine (NAG), nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), silver nitrate, bovine serum albumin (BSA), goat anti-rabbit immunoglobulin G-isothiocyanate conjugate (FITC-IgG), $\propto_2$-macroglobulin antibody (from human plasma) and protease free sucrose were purchased from Sigma Chemical Corporation, (St. Louis, MO.). $\propto_2$-macroglobulin (from human plasma) was obtained from Fluka. Concanavalin A (con A)-sepharose,

wheat germ agglutinin (WGA)-sepharose 6MB and diethylaminoethyl (DEAE) cellulose-sephadex were purchased from Pharmacia Fine Chemicals, (Uppsala, Sweden). *Staphylococcus aureus V8* protease was purchased from Pierce Chemical Company, (Rockford, Ill). [^{3}H]azidopine (40 Ci/mmole), was obtained from Amersham (Oakville, Ontario). Nitrocellulose (0.22 µm) was purchased from Schliecher and Schuell Inc., (Keene, NH). Centricon-30 ultrafiltration concentrators were purchased from Amicon Corporation, (Danvers, MA). All other chemicals were of analytical grade or the highest grade available.

Anti-peptide antibodies to dystrophin from skeletal muscle were a generous gift from Dr. Paul Holland, Neurological Institute, McGill University (Montréal, Québec).

2.2 Membrane Preparations

2.2.1 Cardiac Sarcolemmal Membrane Preparation:

Sarcolemmal enriched membrane fractions derived from porcine myocardium were prepared essentially as previously described (Murphy, 1990). All manipulations were performed at 4°C and all buffers contained the following protease inhibitors; 0.1 mM PMSF, 1.0 µM pepstatin A, 0.75 mM benzamidine, 1.0 mM iodoacetamide. Approximately, 220 g of fresh or frozen porcine ventricular muscle was minced in an *Oster* food processor with 4 x 2 s pulses, aliquoted into 4 equal portions and further homogenized in a Waring blender (max setting) for 3 x 10 s each, in 110 ml of 35 mM Tris-HCl, pH 7.8 containing 20 mM disodium pyrophosphate (buffer A) . The aliquots were then centrifuged at 3,800 x g for 10 min in order to remove red blood cells, nuclei, myofibrils and cellular debris. The supernatant was aspirated and discarded and each pellet was then resuspended in 100 ml of buffer A and homogenized ,

2 x 20 s (20 s between pulses) using a Brinkman Polytron (PT-20) at setting 6. This was then centrifuged as above. The supernatant was aspirated and saved and the pellet was re-extracted two more times as above. The supernatants were pooled and centrifuged at 9,800 x g for 10 min to remove mitochondria. The supernatant was filtered through 2 layers of gauze and centrifuged at 100,000 x g for 50 min. These microsomal pellets which contained both light and heavy membranes were resuspended in 160 ml of 10 mM Tris-HCl, pH 7.4 containing 0.25 M sucrose (buffer B) and layered (10 ml) over a 12 ml solution containing 0.9 M sucrose and 10 mM Tris-HCl, pH 7.4. After centrifugation for 40 min at 100,000 x g in Beckman Ti 42.1 and Ti 60 rotors (16 tubes), membranes at the 0.25/0.9 M interface were aspirated, diluted with 2 volumes of 10 mM Tris-HCl, pH 7.4 (buffer D) and sedimented by centrifugation at 100,000 x g for 45 min. The sedimented membranes which represented the light membranes (enriched in sarcolemma) were resuspended in buffer D at a concentration of 3 to 9 mg/ml, quick-frozen in liquid nitrogen and stored at -70 °C.

For crude membrane preparations, the above was repeated and stopped before the sucrose gradient centrifugation step. In some studies, muscle was homogenized in buffer A and this crude homogenate then analyzed.

2.2.2 Fast-Twitch Skeletal Muscle T-tubule/Sarcolemmal Membrane Preparation:

Sarcolemmal enriched membrane fractions derived from rabbit skeletal muscle were prepared essentially as previously described (Tuana, 1987). Two to 4 kg white New Zealand rabbits were sacrificed by cervical dislocation and the white fast-twitch back muscle was excised and placed on ice. 120 g aliquots were minced in an *Oster* food processor with 4 x 2 pulses and further

homogenized in a Waring blender (max setting) for 20 s in 220 ml of 50 mM Tris-HCl, pH 7.8 containing 20 mM sodium pyrophosphate (buffer A), including protease inhibitors as in cardiac muscle preparations. The membranes were further homogenized using a Brinkman Polytron (PT-20) at setting 6 , 2 x 20 s (20 s between pulses) and centrifuged at 3,800 x g for 10 min in order to remove red blood cells, nuclei, myofibrils and cellular debris. The supernatant was aspirated and the pellets were resuspended in 100 ml of buffer A each and re-homogenized, 2 x 20 s with the polytron and centifuged as above. The supernatant was again saved and the pellet was re-extracted as above. The resultant three supernatants were pooled and centrifuged at 9,800 x g for 10 min to remove mitochondria. The supernatant was decanted, filtered through 2 layers of gauze and centrifuged at 100,000 x g for 50 min to pellet the crude microsomes. Microsomes were subjected to sucrose gradient centrifugation as in the cardiac sarcolemmal preparation (section 2.2.1). Membranes at the 0.25/0.9 M interface were aspirated, diluted with 3 volumes of ice-cold buffer D containing 150 mM KCl and sedimented by centrifugation at 100,000 x g for 45 min. The sedimented membranes were resuspended in buffer D at a concentration of 5 -10 mg/ml and quick-frozen in liquid nitrogen and store at -70°C.

For crude membrane preparations, the above was repeated and stopped before the sucrose gradient centrifugation step. In some studies, muscle was homogenized in buffer A and this crude homogenate then analyzed .

2.3 Partial Purification of the Dihydropyridine Receptor from Cardiac Muscle

Purification was carried out essentially as previously described (Tuana, 1988). All manipulations were carried out at 4°C. Porcine cardiac sarcolemmal

membranes (approx. 40 mg) were thawed and diluted to 2 mg/ml in 50 mM Tris-HCl, pH 7.4 containing the protease inhibitors 0.1 mM PMSF and 1.0 μM pepstatin A (buffer A). Membranes were prelabelled with a saturating concentration of [^{3}H]azidopine (5.0 nM) for 90 min at 4°C to allow sufficient time for association of drug with receptor, in the presence or absence of 1.0 μM (±)PN200-110. Membranes were then sedimented by centrifugation at 120,000 x g for 30 min and resuspended in buffer A containing 0.25% BSA and re-pelleted as above. Membranes were again resuspended in buffer A at 2 mg/ml poured into uncovered 10 x 13 cm Petri dishes and irradiated on ice for 10 min with a hand-held UVSL-56 lamp at a distance of 2 cm. Photolysed membranes were sedimented as above and the supernatant containing unbound drug was discarded and membranes resuspended at 2 mg/ml in 50 mM Tris-HCl, pH 7.4 containing the protease inhibitors 0.75 mM benzamidine and 1.0 mM iodoacetamide (buffer B). The membranes were solubilized at 1 mg/ml with 1% digitonin by the addition of an equal volume of 2% digitonin in buffer B. The mixture was rotated, end over end, for 30 min to solubilize the membranes and insoluble material removed by centrifugation at 120,000 x g for 30 min. Solubilized material contained in the supernatant was removed and kept for purification studies.

Soluble receptor was applied by gravity to a 14 ml DEAE-A 25 column (1.8 x 5.5 cm) pre-equilibrated in 50 mM Tris-HCl, pH 7.4 containing 1.0% digitonin and a full complement of protease inhibitors. Unbound protein and [^{3}H]drug was removed by extensive washing of the column with 50 mM Tris-HCl, pH 7.4 containing 0.1% digitonin and a full complement of protease inhibitors (buffer C). The column was developed with a linear (0.0 - 0.3 M) gradient of NaCl in buffer C, at a flow rate of approximately 45 ml/hr. Approximately 2 ml fractions were collected and aliquots were mixed with 2.0 ml

scintillation cocktail and monitored for [^{3}H]azidopine binding in a Beckman LS 7900 liquid scintillation counter. Peak fractions were pooled and concentrated to a final volume of approximately 2 ml by centrifugation in centricon concentrators at 2,000 x g in a Beckman J-21 rotor. Five to 20% linear sucrose gradients in buffer C (40 ml) were poured by gravity in 42 ml quick-seal polypropylene centrifuge tubes at approximately 4 ml/min using a 60 ml gradient former (30 ml each side). The formed gradients were stored at 4 °C until use. The concentrated DEAE sample was layered onto the surface slowly using a 1 ml syringe with a 22-gauge needle attached. The tube was heat-sealed as per manufacturers instructions and balanced against another gradient. The gradient was centrifuged in a V-Ti 50 vertical rotor at 210,000 for 130 min. The centrifuge was programmed for minimum acceleration until maximum speed was reached and for minimum deceleration from 10,000 rpm to stop. Following centrifugation, the bottom of the tube was punctured with an 18-gauge needle and the gradient fractionated. Aliquots were mixed with scintillation fluid and monitored for [^{3}H]azidopine binding. Peak [^{3}H]azidopine binding fractions were pooled, combined with 1.25 ml of WGA-sepharose pre-equilibrated in buffer C containing 0.3 M NaCl and the mixture was rotated for 1-2 hr at 4 °C. The mixture was packed into a 1.0 x 10 cm column and unbound protein and label was removed by extensive washing at 70 ml/hr with buffer C containing 0.3 M NaCl. Bound proteins were specifically eluted with buffer C containing 0.3 M N-acetyl-D-glucosamine. Aliquots of column eluate were monitored for [^{3}H]azidopine binding and peak fractions were pooled and concentrated by centrifugation as described above. In order to determine the molecular mass of the labelled peptide(s), concentrated eluted protein from the WGA-sepharose lectin column was subjected to SDS-PAGE and gel lanes sliced into 2 mm strips followed by overnight digestion in 30% H_2O_2 at 60°C.

Scintillation fluid was then added to the vials and the samples allowed to equilibrate for 72 hr prior to measuring radioactivity (10 min/vial).

2.4 Purification of the 360 kDa Protein from Cardiac Muscle

All manipulations were carried out at 4°C. Porcine cardiac sarcolemmal membranes (approx. 40 mg) were thawed and diluted to 2 mg/ml in 50 mM Tris-HCl, pH 7.4 containing the protease inhibitors 0.1 mM PMSF and 1.0 μM pepstatin A. The membranes were solubilized at 1 mg/ml with 1% NP-40 by the addition of an equal volume of 2% NP-40 in 50 mM Tris-HCl, pH 7.4, containing the protease inhibitors 0.75 mM benzamidine and 1.0 mM iodoacetamide and the mixture was rotated, end over end, for 30 min to solubilize the membranes. Insoluble material was removed by centrifugation at 120,000 x g for 45 min. Greater than 90% of membrane proteins were solubilized by this procedure. Solubilized material contained in the supernatant was removed and saved.

Soluble protein was applied by gravity to a 3 ml con A-sepharose column (1.0 cm x 10 cm) pre-equilibrated in 50 mM Tris-HCl, pH 7.4 containing 0.1% NP-40, 0.5 M NaCl and protease inhibitors (buffer A). Unbound protein was removed by extensive washing of the column with buffer A. The column was incubated overnight with buffer A containing 250 mM methyl α-D-mannopyranoside and eluted the following day. Eluted fractions were concentrated to a final volume of approximately 2-3 ml by centrifugation in centricon microconcentrators at 2,000 x g in a Beckman J-21 rotor. Five to 20% linear sucrose gradients were prepared as described in Section 2.3. The formed gradients were stored at 4°C until use. The concentrated sample (2-3 ml) was layered onto the surface slowly using a syringe with a 22-gauge needle attached. The tube was heat-sealed as per manufacturers instructions and balanced against another gradient. The gradient was centrifuged in either

Beckman VC53 or V-Ti 50 vertical rotors at 210,000 x g for 165 min. The centrifuge was programmed for minimum acceleration until maximum speed was reached and for minimum deceleration from 10,000 rpm to stop. Following centrifugation, the bottom of the tube was punctured with an 18-gauge needle and the gradient fractionated. Fractions were then subjected to SDS-PAGE and visualized by silver staining. Fractions containing pure protein were pooled, concentrated by centrifugation as described above, quick-frozen in liquid nitrogen and stored at -70°C.

2.5 Partial Purification of Dystrophin from Cardiac Muscle

Partial purification of dystrophin was carried out essentially as described in Section 2.4. Porcine cardiac membranes (40 mg) were solubilized with 1% NP-40 in a buffer containing 0.5 M NaCl, 50 mM Tris, pH 7.4, 0.1 mM PMSF, 1.0 µM pepstatin, 0.75 mM benzamidine and 1.0 mM iodoacetamide (buffer A) at a protein concentration of 1 mg/ml. Solubilized membranes were applied to a 3 ml con A-sepharose column pre-equilibrated in buffer A containing 0.1% NP-40 and circulated overnight at 4°C. Flow throughs were collected and columns then extensively washed with buffer A. The column was then eluted with three column volumes of 0.3 M methyl∝-D-mannopyranoside in buffer A containing 0.1% NP-40 and 0.75 ml fractions were collected. Samples were concentrated and frozen at -70°C until use.

2.6 Subcellular Fractionation of Cardiac and Skeletal Muscle Membranes

Porcine cardiac and rabbit skeletal (fast-twitch) membranes were isolated as described in sections 2.2.1 and 2.2.2. Low speed supernatants were pooled and centrifuged at 100,000 x g for 50 min in a Beckman Ti 42.1

rotor. The supernatant represents the soluble (cytosolic) fraction and the pellet, the microsomal fraction. Microsomal pellets were resuspended in a homogenization buffer containing 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose and protease inhibitors and fractionated on a 30% (0.9 M) discontinuous sucrose gradient. Membranes at the 0.25/0.9 M interface represented the light membrane fraction enriched in sarcolemma and the pellet, the heavy membrane fraction enriched in sarcoplasmic reticulum, transverse tubules and terminal cisternae.

2.7 KCl Wash of Membranes

Cardiac sarcolemmal membranes (30 mg) were incubated with a solution containing 1.1 M KCl, 50 mM Tris-HCl, pH 7.4 and a protease inhibitor mix of 0.1 mM PMSF, 1.0 µM pepstatin A, 0.75 mM benzamidine and 1.0 mM iodoacetamide to give a final KCl concentration of 0.65 M. This concentration of KCl has been shown to remove peripherally bound sarcolemmal proteins leaving integral proteins in the sarcolemma. This mixture was rotated for 1 hr at 4°C and then centrifuged at 100,000 x g for 45 min in a Beckman Ti 42.1 rotor to pellet down membranes. Supernatants were aspirated and saved. The pellet was washed by resuspending in a buffer containing 10 mM Tris, pH 7.4 (buffer D) and protease inhibitor mix and centrifuged as above. The supernatant was discarded and the pellet resuspended in buffer D. Samples were saved and stored at -70°C.

2.8 Electrophoresis and Related Techniques

2.8.1 SDS Polyacrylamide Gel Electrophoresis:

Protein samples were processed for SDS-PAGE by heating at 90°C for 1 min in a solution containing 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS

and 0.005% bromphenol blue (sample loading buffer), nonreducing conditions, or in sample loading buffer containing 10 mM DTT, reducing conditions. Protease inhibitors were added in order to prevent protein degradation. Samples were cooled and applied to uniform concentration polyacrylamide gels using the discontinuous buffer system as described by Laemmli (1970) or uniform concentration porous polyacrylamide gels using the discontinuous buffer system as described by Doucet *et al* (1990) on a Mini Protean II Gel apparatus (Bio-Rad) unless otherwise stated.

2.8.2 Silver Staining of SDS-Polyacrylamide Gels:

Gels were stained using a modified protocol based on the original procedure of Morrisey (1981). Following electrophoresis, gels were fixed in 30% ethanol and 10% acetic acid for 20 min in a polystyrene dish with gentle rocking. The gel was subsequently washed twice for 10 min in 10% ethanol to remove the acetic acid and residual SDS and electrophoresis buffers, followed by 3 washes of 10 min each with distilled H_20. The gel was then incubated for 20 min in 35 µm DTT dissolved in distilled H_20 followed by incubation in 0.1% silver nitrate dissolved in distilled H_20 for 20 min. The gel was rinsed twice in distilled water and developed in a solution of 3.0% sodium carbonate containing 0.05% formaldehyde. The development was stopped by removing the developer and adding a solution of 1% acetic acid. The gel was subsequently washed and stored in distilled H_2O.

2.8.3 Coomassie Brilliant Blue Staining of SDS-Polyacrylamide Gels:

Following electrophoresis, proteins were fixed for 20 min using a solution containing 30% ethanol and 10% acetic acid with gentle shaking. The gel was then incubated with 0.125% Coomassie Brilliant Blue R-250 dissolved in 50%

methanol and 4.6% acetic acid for 30 min. Protein bands were visible following destaining of the gel with 7.5% acetic acid and 5% methanol over a period of 2 hrs or longer if necessary. Gel was washed frequently dur ng this time using destaining solution. Destaining was accelerated by placing kimwipes into the solution, which aided destaining by soaking up the residual stain.

2.8.4 Determination of Molecular Mass of Proteins:

The apparent molecular mass of SDS-PAGE resolved proteins was estimated graphically by plotting the relative mobility (R_f) vs the log molecular weight of known protein standards visualized by silver staining or by using pre-stained molecular weight standards (Sigma). The apparent molecular mass (Mr) of unknown proteins were estimated by determining the R_f from the centre of the given band and interpolating this value from the standard curve.

2.8.5 Determination of Protein Concentrations:

Protein concentrations were estimated by the method of Bradford (1976) using BSA as a standard (Bio-Rad Protein Assay).

2.8.6 Proteolytic Maps:

Proteolytic digestions were carried out as described by Cleveland *et al* (1977). Essentially, con A-purified-360 kDa protein and α_2M were subjected to SDS-PAGE on a 7% homogenous gel (13 x 16 cm). The gel was stained with Coomassie Brilliant Blue R-250 to identify the bands, destained and bands excised. Excised bands were then placed in sample wells of a second gel (16% acrylamide, 13 x 16 cm) and overlayed with a buffer containing 0.1% SDS, 20% glycerol and 200 ng *S. aureus V8* protease. Samples were electrophoresed into the stacking gel and the power

subsequently turned off for 5 to 10 min. The power was then restored and the gel run at 50 V overnight until the tracking dye reached the bottom of the gel. The gels were then stained with silver to visualize the bands.

2.8.7 Transfer of Proteins to Nitrocellulose Membranes:

Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose as described by Towbin *et al* (1979). Following SDS-PAGE, gels were equilibrated in transfer buffer containing 25 mM Tris, 150 mM glycine, 20% methanol v/v for 20 min to remove electrophoresis buffer salts and detergents. Gels were then placed on a piece of Whatman 3M filter paper and overlayed with a 0.22 µM nitrocellulose sheet. A second piece of filter paper was placed over the nitrocellulose. These were then positioned between two sponges, placed in plastic supports and soaked in transfer buffer. Proteins were electrophoretically transferred for 1 hr at 4°C at 100 mA in a Hoefer Scientific Trans-Phor Model TE42 transfer apparatus.

2.8.8 Ponceau S. Staining of Proteins Transferred to Nitrocellulose Membranes:

Following transfer of proteins to nitrocellulose, the membrane was briefly incubated with the reversible stain, Ponceau S., for 30 s followed by 3 rinses in distilled H_20 in order to visualize transferred protein bands. The success of the transfer was thereby assessed. Stain was removed by rinsing nitrocellulose membrane in TBST.

2.8.9 Western Blotting:

Nitrocellulose membranes with transferred proteins were blocked in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl (TBS) containing 5% skim milk powder

(BLOTTO) for 1 hr at RT with moderate shaking. This blocked non-specific binding and reduced background. BLOTTO was removed by three 5 min washes in TBS containing 0.1% Tween-20 (TBST). The membrane was then incubated with various dilutions of primary antibody in TBST for 1-2 hrs with gentle rocking. Unbound primary antibodies were removed by three 5 min washes in TBST. The membrane was then incubated in a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG second antibody in TBST for 1 hr at RT with gentle rocking. Unbound second antibody was removed by three 5 min washes in TBST. Immunoreactive bands were visualized by the addition of an alkaline phosphatase buffer, 100 mM Tris-HCl, pH 9.5, 10 mM NaCl and 5 mM $MgCl_2$, containing 0.33 mg/ml NBT and 0.16 mg/ml BCIP, until a colour reaction was observed. The colour reaction was stopped by washing the membranes in distilled H_2O.

2.8.10 Lectin Overlays:

Nitrocellulose membranes with transferred proteins were blocked in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl (TBS) containing 2.5% BSA for 1 hr at RT with moderate shaking. This blocked non-specific binding and reduced background. Excess BSA was removed by three 5 min washes in TBS containing 0.1% Tween-20 (TBST). Membranes were then incubated in TBST containing either horseradish peroxidase-conjugated-WGA lectin or -con A lectin at 1 µg/ml for 1 hr at RT with gentle shaking. Unbound lectin was removed with three 10 min washes with TBS. Tween-20 was omitted from these steps as it inhibits the subsequent colour development reaction. Bound horseradish peroxidase-conjugated lectin was visualized as follows: solid 4-chloro-1-naphthol was dissolved in ice-cold methanol at a concentration of 3.0 mg/ml (Sol A). Next, 0.024 ml ice-cold 30% H_2O_2 was added to 40 ml of 10 mM

Tris-HCl (pH 7.4) (Sol B). Sol A and Sol B were combined in a ratio of 1:5 and added to the membrane. Development reaction was terminated by transferring the membrane to distilled H_2O.

2.9 Production of Polyclonal Antibodies

2.9.1 Immunization Protocol:

A New Zealand white rabbit (2-2.5 kg) was used as the host animal for polyclonal antibody production. Prior to immunization, rabbit was bled for preimmune control serum. For immunization, 50 µg of protein was emulsified with an equal volume of Freund's complete adjuvant by forcing the mixture back and forth between two 1 ml syringes connected by way of a three-way Luer lock. The components were mixed until a thick white suspension was produced. The resultant mixture (0.25 ml) was immediately injected intramuscularly into the hind leg of the animal. This was designated day 0. At day 14, blood samples were taken to monitor antibody production and the rabbit was boosted with another 50 µg of purified protein emulsified in Freund's incomplete adjuvant. Weekly blood samples were taken and antibody production monitored by Western blotting.

2.9.2 Preparation of Crude Antiserum from Whole Blood:

Each sample of whole blood was incubated at 4°C for 16 hours to allow complete clotting. The blood was subsequently centrifuged at 5,000 x g for 10 min to sediment red blood cells. The serum was collected and centrifuged again at 5,000 x g for 10 min to remove any residual red blood cells. Serum was decanted, aliquoted and stored at -70°C.

2.9.3 Purification of α_2-Macroglobulin Antibodies:

Purification of α_2M antibodies was carried out essentially as previously described (Tuana, 1991). Approximately 5 mg of α_2M (Fluka) was loaded onto a 2 cm^2 nitrocellulose filter. The nitrocellulose was then blocked for 1 hr at RT with 3% BSA in PBS at 250 µl/sq cm. Nitrocellulose was then incubated with a 1:50 dilution of affinity purified IgG fraction of α_2M antibodies (Sigma) in 3% BSA/PBS for 5 hrs at RT or 16 hrs at 4°C with gentle shaking. Following incubation, the antibody solution was aspirated off and the nitrocellulose filter rinsed with 0.15 M NaCl for 15 min at RT, followed by a further 15 min in PBS. Pure antibody was eluted from the nitrocellulose square with a solution containing 0.2 M glycine (pH 2.8) and 1 mM EGTA. Eluted antibody was transferred to a small test tube and the elution buffer neutralized with 0.1 volume of 1 M Tris, pH 9.5. Pure antibody was stored in aliquots at -70°C.

2.9.4 Absorption of α_2-Macroglobulin Antibodies:

α_2M antibodies (10 µl) were incubated with excess α_2M (200 µl) in 1% BSA/PBS overnight at 4°C. After centrifugation at 100,000 x g for 3 hrs, the supernatant solution was aspirated and stored at -70°C. The absorbed antibody was used as a further control in immunofluorescence studies.

2.10 Immunohistochemical Localization

2.10.1 Preparation of Cardiac Tissue Sections:

Fresh cardiac muscle was obtained from a slaughterhouse, cut into approximately 1 cm^3 squares and transported on dry ice. Muscle cubes were then placed on a thin cardboard strip treated with freezing agent and tissue section was quick-frozen with a blast of CO_2. Frozen tissue was then placed in a pre-cooled cryostat (Microm, Heidelberg). Transverse and longitudinal

sections 8 µm thick were collected on pre-treated glass slides (Fisher Brand) which allowed a good adherence of tissue slices to the slides. Slides were stored at -70°C until use.

2.10.2 Immunofluorescence Labelling of Cardiomyocytes:

Immunofluorescence labelling was carried out essentially as described (Harlow, 1988). Freshly isolated cells (Désilets, 1986) were washed twice by centrifugation at 400 x g for 5 min each and resuspended in PBS. Cell pellet was resuspended in PBS at 10^5 cell/ml. Cells were then added to poly-L-lysine coated coverslips and incubated at 37°C for 2 hrs. Poly-L-lysine enhanced the adherence of cells to the coverslips. Cells used for permeabilization studies were rinsed with PBS, fixed in 3.7% formaldehyde in PBS for 20 min, washed twice with PBS and permeabilized with 0.2% Triton X-100 in PBS for 15 min at RT. All coverslips were washed with 3 washes of PBS. Preparations were then incubated with various dilutions of primary antibody in PBS containing 3% BSA for 45 min in a humid chamber at 37°C. Coverslips were then washed 3 times with PBS and incubated with goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate conjugate (FITC-IgG) secondary antibody at a dilution of 1:200 for 30 min in a humid chamber at 37°C. Coverslips were washed 3 times with PBS and fixed with 3.7% formaldehyde in PBS for 20 min. Finally, coverslips were rinsed twice in PBS and mounted in glycerol-PBS (1:1 v/v) with 0.1% *p*-phenylenediamine (to retard fading of fluorescence). Some control experiments were performed with both secondary antibody alone and antibody adsorbed with antigen.

Preparations were observed with a Leitz Ortholux 11 fluorescent microscope equipped with a 200-W high-pressure lamp and a Ploemopack 11

incident light illuminator equipped with an I-filter block. Photographs were taken with Kodak Tri-X pan films (400 ASA).

2.10.3 Immunofluorescence Labelling of Tissue Sections:

Labelling was carried out essentially as described for cardiomyocytes with the following alterations. Slides were thawed and sections rehydrated with PBS. Tissue was fixed in 4% paraformaldehyde for 2 min, washed with PBS and permeabilized with 1% NP-40 in PBS for 5 min. Following incubation with primary and secondary antibodies as described for cardiomyocytes, slides were mounted and observed.

3.0 RESULTS

3.1 Partial Purification of the Dihydropyridine Receptor

In order to attempt to enrich and identify the components of the DHP receptor from cardiac muscle, sarcolemmal membranes were used as the starting material for the purification. Sarcolemmal enriched membranes were obtained following the subfractionation of porcine heart ventricles by differential centrifugation and discontinuous sucrose density gradient centrifugation as described in Materials and Methods. This membrane fraction had previously been found to be enriched in sarcolemmal enzyme markers such as Na^+-K^+ ATPase, adenylate cyclase and DHP receptors (Tuana, 1987). Sarcolemmal enriched membranes suspended at a concentration of 2.0 mg/ml in 50 mM Tris-HCl, pH 7.4 containing protease inhibitors were photoaffinity labelled with a saturating concentration of [^{3}H]azidopine (5.0 nM). The labelled membranes were then solubilized with 1% digitonin at a protein concentration of 1 mg/ml for 30 min and insoluble membranes removed by centrifugation (Tuana, 1990). The bound label was then followed through various purification steps. Solubilized membranes were applied to a DEAE-sephadex column, a column which binds proteins via charge exchange. Unbound protein and free unbound drug was removed by washing the column with 50 mM Tris-HCl, pH 7.4, containing 0.1% digitonin and a full complement of protease inhibitors. The column was eluted with a 0.0-0.3 M linear NaCl gradient in the above buffer. Aliquots of the eluted fractions were monitored for [^{3}H]azidopine binding by liquid scintillation counting. Binding material extracted from cardiac muscle was eluted as a relatively broad peak between 0.075-0.13 M NaCl. The peak [^{3}H]azidopine binding fractions were then pooled, concentrated by centrifugation and subjected to centrifugation through a 5-20% linear sucrose gradient. Sucrose density gradient centrifugation separates proteins on the

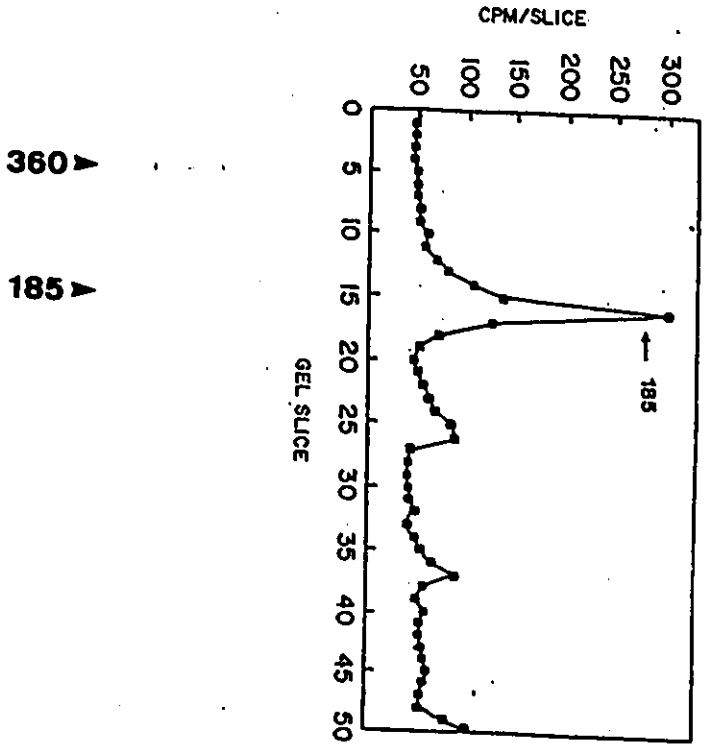
basis of size, shape and density and is often used to separated high molecular weight proteins from lower molecular weight proteins. The sucrose gradient was fractionated by collecting fractions from the bottom of the tube. Aliquots of the fractions were monitored for [^{3}H]azidopine binding. The [^{3}H]azidopine binding material was found to sediment as a relatively sharp peak and these fractions were pooled and mixed with 1.25 ml of WGA-sepharose and incubated for 1-2 hr at 4°C. WGA-sepharose binds glycoproteins having N-acetyl-D-glucosamine sugar residues. To prevent nonspecific adsorption of proteins to the sepharose beads, 0.3 M NaCl was included in the buffers. Unbound protein was removed by extensively washing the column in high salt buffer and specifically bound proteins were eluted with 0.3 M NAG. Upon addition of 0.3 M NAG [^{3}H]azidopine binding material was eluted as a single peak. Sample was analyzed for protein content by SDS-PAGE and is shown in Fig. 3A. A high molecular weight protein of Mr 360,000 and the α_1 subunit (Mr 185,000) of the DHP receptor comprised the final purification fraction (Murphy, 1990). Following cut and slice analysis of the SDS-gel, the [^{3}H]azidopine label was incorporated in a protein of Mr 185,000 (Fig. 3B). No radioactivity was incorporated in the protein band corresponding to Mr 360,000.

3.2 Purification of the 360 kDa Protein

In order to study the structure of the 360 kDa protein, sarcolemmal membranes were again used as the starting material for the purification as in Section 3.1. A simplified and efficient purification protocol for the 360 kDa protein was developed. Sarcolemmal enriched membranes from porcine ventricles, suspended at a concentration of 2.0 mg/ml in 50 mM Tris-HCl, pH 7.4 containing protease inhibitors, were solubilized with 1% NP-40 at a protein concentration of 1 mg/ml for 30 min and insoluble membranes were removed by

Figure 3: Partial purification of the dihydropyridine receptor from cardiac sarcolemmal membranes:

Porcine cardiac sarcolemmal membranes were covalently labelled with [^{3}H]azidopine and solubilized with 1% digitonin. Labelled receptor was followed on a DEAE ion exchange column, sucrose density gradient centrifugation and a WGA lectin affinity column, as described in "Materials and Methods". Peak [^{3}H]azidopine binding fractions, were subjected to 7% SDS-PAGE and gels were either silver stained (A) or cut into 2 mm slices, digested in H_2O_2 and radioactivity monitored by scintillation counting. Counts (cpm) / slice are shown in (B). Positions of the α_1 subunit (185 kDa) and 360 kDa protein are shown.

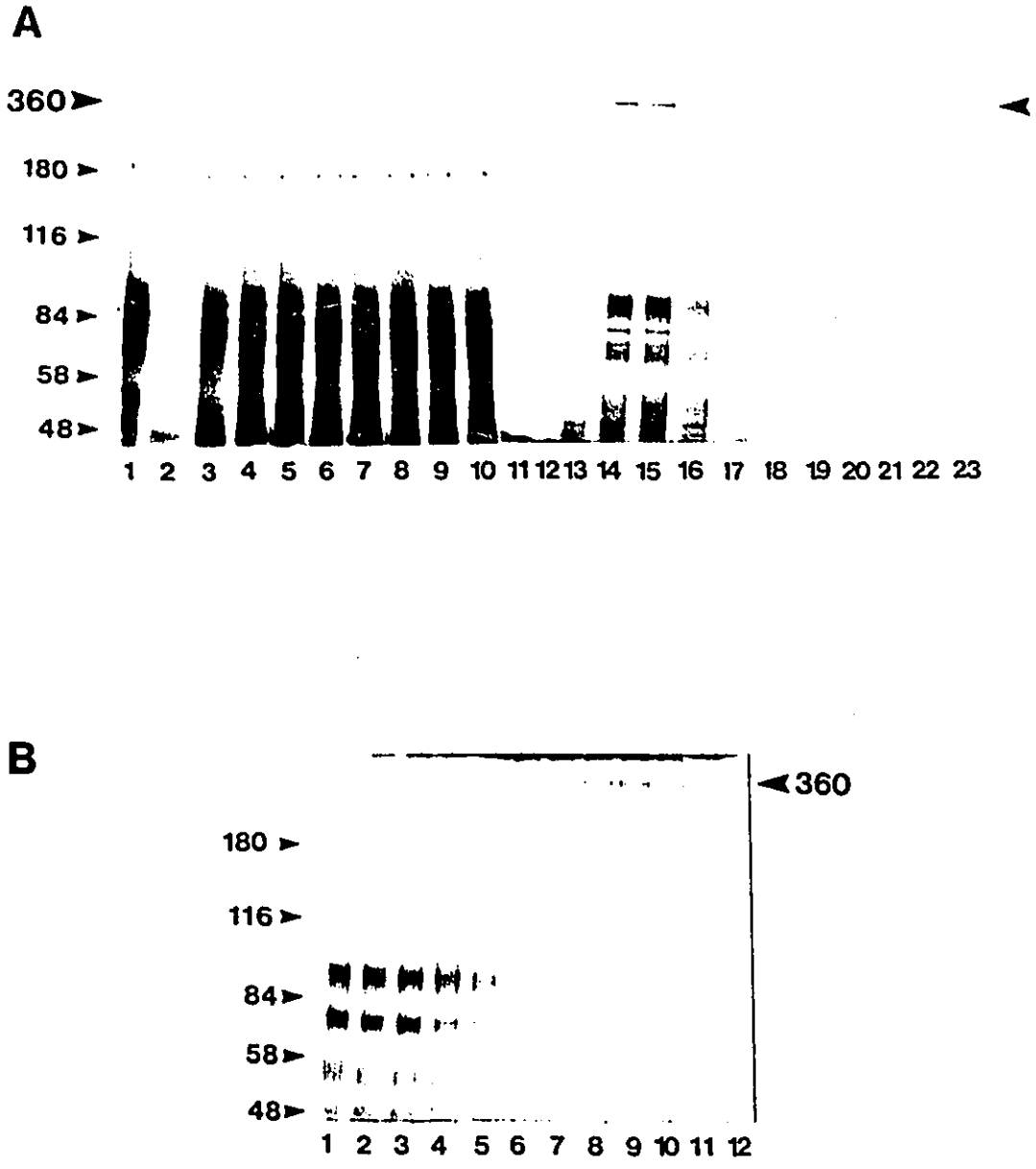

360
185
CPM/SLICE
50
100
150
200
250
300
0
5
10
15
20
25
30
35
40
45
50
GEL SLICE
185
A
B

centrifugation. Soluble protein was then applied to a 3 ml con A-sepharose lectin column. Con A is a lectin which binds mannose sugars with high affinity. Previously, WGA lectin (which binds N-acetyl-D-glucosamine sugars with high affinity) was used in order to isolate this protein. Because of this varied sugar specificity, con A lectin column was used to aid in the purification of the 360 kDa protein from other glycoproteins present. Nonspecifically bound proteins were removed by washing the column with a high salt buffer containing 50 mM Tris-HCl, pH 7.4, containing 0.1% NP-40, 0.5 M NaCl and a full complement of protease inhibitors. Bound proteins were then specifically eluted with the sugar MDM (0.25 M). Fractions were analyzed for protein content by SDS-PAGE. Polyacrylamide gels were stained with silver to visualize the proteins. Fig. 4A shows the polypeptide composition of the column flow-through, wash fractions and material specifically eluted from the lectin column. The 360 kDa polypeptide was >90% adsorbed by the con A column as indicated by its absence in the flow through fractions. The 360 kDa protein is visible following elution with MDM. Only following overnight incubation with elution buffer was elution of the 360 kDa protein successful. Scaling up the procedure led to complete loss of column resolution. When larger amounts of protein were purified, a series of 3 ml con A columns were used. Ratio of SL membranes to con A-sepharose was 40 mg of SL protein/3 ml of con A-sepharose. Con A lectin column separated various contaminating glycoproteins away from the 360 kDa protein which were not separated on the WGA lectin column. The eluted fractions were then pooled, concentrated by centrifugation and separated through a 5 - 20% (w/v) linear sucrose gradient. The sucrose gradient was fractionated by collecting fractions from the bottom of the tube. Proteins contained in these fractions were visualized by silver staining following SDS-PAGE and are shown in Fig. 4B. The 360 kDa protein was purified away from

Figure 4: Purification of the 360 kDa protein from porcine cardiac sarcolemmal membranes:

(A) Binding of the 360 kDa protein to a lectin affinity column. Porcine cardiac sarcolemmal membranes were solubilized with 1% NP-40, followed on a con A-sepharose lectin affinity column and adsorbed proteins specifically eluted with 0.25 M MDM, as described in "Materials and Methods". Proteins were subjected to 7% SDS-PAGE and visualized with silver stain. Lane numbers indicate fraction numbers: lane 1, NP-40 solubilized SL membranes; lanes 2-10, flow through fractions; lanes 11-12, wash fractions; lanes 13-23, eluted fractions. The positions of the molecular weight standards and 360 kDa protein are indicated.

(B) Purification of the 360 kDa protein on a sucrose density gradient. Eluted fractions from the con A column containing the 360 kDa protein (see above), were sedimented through a 5-20% linear sucrose density gradient. Following gradient fractionation, every third fraction was subjected to 7% SDS-PAGE and visualized with silver stain as described in "Materials and Methods". Fraction numbers 1-12 correspond to top-bottom of the gradient. The positions of the molecular weight standards and 360 kDa protein are indicated.


A
360
180
116
84
58
48
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
B
360
180
116
84
58
48
1 2 3 4 5 6 7 8 9 10 11 12

contaminating proteins by this method, the protein being purified to almost homogeneity (>95% pure). A yield of ~25 µg from 40 mg of SL (~0.5-1 µg/SL membranes) was recovered. Samples were then quick frozen in liquid nitrogen and stored at -70°C for biochemical characterization.

3.3 Biochemical Characterization of the 360 kDa Protein

3.3.1 Subunit Composition of the 360 kDa Protein:

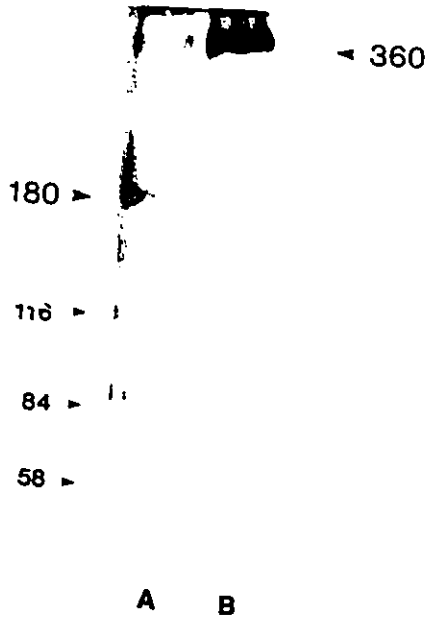
To determine whether or not the 360 kDa protein was a monomer, migrating at a molecular weight corresponding to 360 kDa following SDS-PAGE, pure protein was incubated with 10 mM DTT in SDS-sample buffer and electrophoresed. DTT is a reducing agent which is known to disrupt disulfide linkages. The reduced and nonreduced forms of the protein following SDS-PAGE are shown in Fig. 5. The 360 kDa protein migrated to a molecular weight 180 kDa (Fig. 5, lane a) following reduction. This suggested that the native 360 kDa protein consists of two peptide chains of 180 kDa linked together by disulfide bonds.

3.3.2 Determination of the Glycoprotein Nature of the 360 kDa Protein:

The ability of the 360 kDa protein to bind to and be specifically eluted from the con A-sepharose lectin column (and WGA-sepharose lectin column from previous studies), suggests that the 360 kDa protein is glycosylated. Glycoproteins are adsorbed by lectin columns via their sugar residues. It has been demonstrated however that nonglycosylated proteins can be specifically eluted from the various lectin columns. This specific elution arisies from the association of these proteins with glycoproteins bound to the column (Campbell,1989). Therefore, in order to determine whether the 360 kDa protein was glycosylated or simply associating with other sarcolemmal glycoproteins,

Figure 5: Subunit composition of the 360 kDa protein:

The 360 kDa protein was resolved under reducing (10 mM DTT) (A) and nonreducing (B) conditions, following SDS-PAGE (7% acrylamide) and visualized with silver stain as described in "Materials and Methods". The position of the 360 kDa protein, the 180 kDa reduced product and molecular weight standards are indicated.


360
180
116
84
58
A
B

lectin overlays were performed. Porcine sarcolemma, protein specifically eluted from the con A-sepharose column and pure 360 kDa protein were separated by SDS-PAGE under both reducing and nonreducing conditions, transferred to nitrocellulose and overlayed with horseradish peroxidase-labeled-con A and -WGA. Resolved proteins were firstly visualized with Ponceau S. stain (Fig. 6A) in order to assess the success of the protein transfer. Note the different protein profiles of each fraction. The nitrocellulose was then stained with the lectins and the results of these overlays are presented in Fig. 6. Both peroxidase-labeled-con A (Fig. 6B) and -WGA (Fig. 6C) stained the 360 kDa protein and the 180 kDa reduced product (Fig. 6B and 6C, lanes 3a and 3b). This indicated that the 360 kDa protein was in fact a glycoprotein and that it contained both mannose and N-acetyl-D-glucosamine sugar residues. Note that many other glycoproteins exist in the SL (Fig. 6B and 6C, lane 1) and in the partially pure preparation (Fig. 6B and 6C, lane 2) and these are absent in the purified 360 kDa protein fraction.

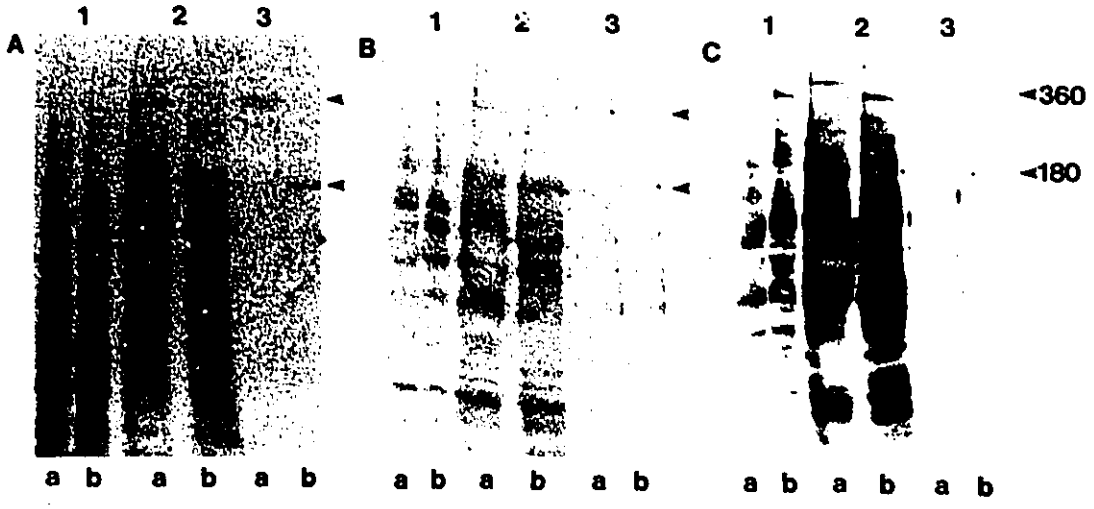
3.4 Western Blot Analysis of the 360 kDa Protein

3.4.1 Production of Anti-360 kDa Protein Antibodies:

Antibodies have been widely used as a tool for the study of the structure and function of many proteins. Therefore, polyclonal antibodies were prepared against the purified 360 kDa protein. A rabbit was chosen as the host animal for immunization due to its large size and correspondingly large blood volume to ensure the collection of sufficient quantities of serum for subsequent experiments. Pure protein was emulsified in complete Freund's adjuvant and injected intramuscularly into the hind leg to initiate a vigorous immune response. Two weeks later, the rabbit was boosted with pure protein emulsified in incomplete Freund's adjuvant.

Figure 6: Lectin binding to the 360 kDa protein:

Porcine cardiac SL (1), protein specifically eluted from con A-sepharose column (2) and pure 360 kDa protein obtained from sucrose gradient centrifugation (3) were subjected to 7% SDS-PAGE, under both nonreducing (a) and reducing (10 mM DTT) (b) conditions as described in "Materials and Methods". The resolved proteins were then transferred to nitrocellulose stained with Ponceau S. (A) and peroxidase-labelled-con A (B) and peroxidase-labelled WGA (C) as described in "Materials and Methods". The 360 kDa protein and 180 kDa reduced product are indicated.


A
1
2
3
a b a b a b
B
1
2
3
a b a b a b
C
1
2
3
a b a b a b
360
180

3.4.2 Specificity of Anti-360 kDa Protein Antibodies:

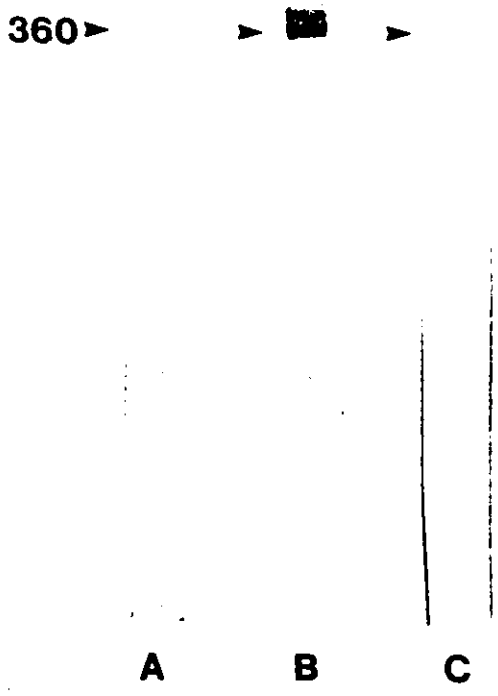
Western blotting was used to monitor the production and specificity of anti-360 kDa antibodies. Prior to immunization, preimmune serum was taken as a control to measure any pre-existing crossreacting antibodies. Serum was prepared from whole blood samples taken at various time intervals as described in "Material and Methods". Crude cardiac SL membranes were separated on SDS-PAGE, transferred to nitrocellulose and immunoblotted with preimmune serum and immune serum at various dilutions as shown in Fig. 7. No reaction was observed with the preimmune serum (Fig. 7A). The presence of circulating antibodies two weeks following boosting are shown in Fig. 7B and 7C, representing dilutions of 1:1,000 and 1:10,000, respectively. Antibody titres as high a 1:16,000 dilution were attained.

3.4.3 Integral and Peripheral Protein Nature of the 360 kDa Protein:

Proteins are classified as either integral, embedded deep within the lipid membrane bilayer or peripheral, loosely bound to the cytoplasmic or extracellular side of the membrane. In order to determine whether the 360 kDa protein was an integral or a peripheral membrane protein, SL membranes were washed with KCl (0.65 M final). This high concentration of KCl is generally capable of dissociating peripherally bound proteins from membranes. Following incubation of SL membranes with 0.65 M KCl and centrifugation, the supernatant, which contained dissociated peripherally bound proteins, was collected. The pellet was resuspended and washed with a buffer containing 50 mM Tris-HCl, pH 7.4 and protease inhibitors. A sample of SL membranes, KCl extracted pellet and the supernatant, were subjected to SDS-PAGE, transferred to nitrocellulose and blotted with anti-360 kDa antibodies (Fig. 8). The

Figure 7: Specificity of anti-360 kDa protein antibodies:

Antibodies against pure 360 kDa protein were raised in rabbits as described in "Materials and Methods". Porcine cardiac membranes were subjected to SDS-PAGE (7% acrylamide) and transferred to nitrocellulose as described in "Materials and Methods". Lane A was immunoblotted with preimmune serum, lane B with immune serum 1:1000 dilution and lane C with immune serum 1:10,000 dilution, as described in "Materials and Methods". Immune serum tested was from 2 weeks following boosting. Position of the 360 kDa protein is indicated.


360
A
B
C

presence of the 360 kDa protein was noted in all samples, with staining appearing slightly stronger in the washed membrane pellet (Fig. 8B) compared to the supernatant (Fig. 8C). This indicated that the 360 kDa may be both peripheral and integral.

3.4.4 Western Blotting of the 360 kDa Protein with Anti-360 kDa Protein Antibodies and Anti-Dihydropyridine Receptor Antibodies:

In order to determine if the 360 kDa protein was purified away from the DHP receptor, samples from the various purification steps in the isolation of the 360 kDa protein were subjected to SDS-PAGE, transferred to nitrocellulose and blotted with both anti-360 kDa antibodies and anti-DHP receptor antibodies. Anti-360 kDa antibodies (Fig. 9A) specifically recognized the 360 kDa protein in fractions from cardiac SL(Fig. 9A, lane a), con A-lectin column (Fig. 9A, lane b) and sucrose gradient (Fig. 9A, lane c). Anti-DHP receptor antibodies recognized the α_1-subunit of the DHP receptor in fractions from the SL (Fig. 9B, lane a) and the con A-lectin column (Fig. 9B, lane b) but not in the pure 360 kDa protein fraction obtained following sucrose gradient centrifugation (Fig. 9B, lane c). This indicated that the 360 kDa protein was purified away from the DHP receptor. Furthermore, the anti-360 kDa antibodies did not crossreact with the α_1-subunit and the anti-DHP receptor antibodies did not crossreact with the 360 kDa protein. This indicated that these proteins were distinct and not related. These blots also demonstrated the advantage of using the con A-sepharose lectin column over the WGA-sepharose lectin column in the purification of the 360 kDa protein. The con A-lectin column enriched the 360 kDa protein (Fig. 9A, lane b) when compared with the SL (Fig. 9A, lane a). The α_1-subunit on the other hand, was not entirely retained by the column, staining

Figure 8: Detection of the 360 kDa protein in cardiac sarcolemma extracted with KCl:

Cardiac SL membranes were washed with KCl (0.65 M final) as described in "Materials and Methods". SL membranes, 35 µg (A), KCl washed SL membranes, 35 µg (B) and supernatant of KCl washed SL membranes, 35 µg (C) were resolved under nonreducing (a) and reducing (10 mM DTT) (b) conditions following SDS-PAGE (7% acrylamide) as described in "Materials and Methods". Immunoblotting of nitrocellulose membranes was carried out as described in "Materials and Methods" using anti-360 kDa protein antibodies. The position of 360 kDa protein and 180 kDa reduced product are indicated.

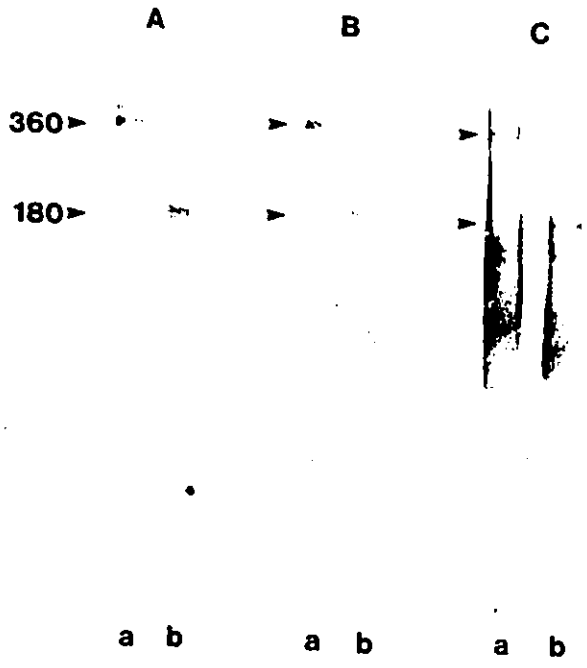
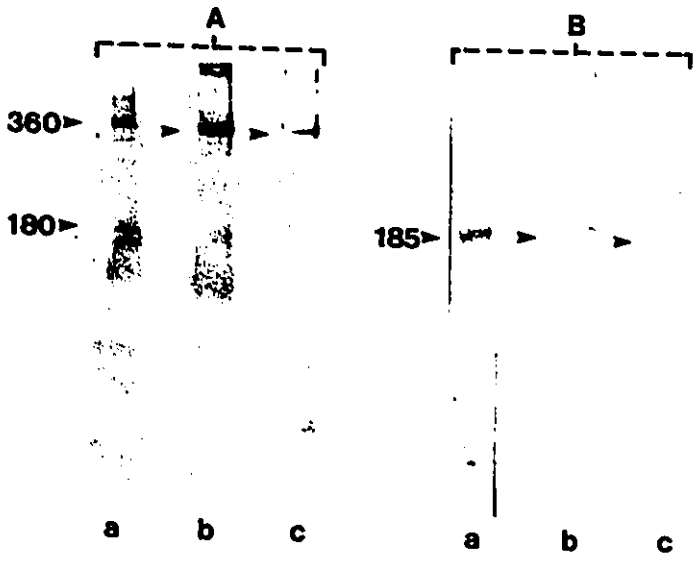

A
B
C
360
180
a b
a b
a b

Figure 9: Immunoblotting of the 360 kDa protein with anti-360 kDa protein antibodies and anti-dihydropyridine receptor antibodies:

Porcine cardiac SL (a), protein specifically eluted from con A-sepharose column (b) and pure 360 kDa protein obtained from sucrose gradient centrifugation (c) were subjected to 7% SDS-PAGE under nonreducing conditions and transferred to nitrocellulose, all procedures as described in "Materials and Methods". (A) represents immunoblotting of nitrocellulose membranes with anti-360 kDa protein antibodies and (B) immunoblotting of nitrocellulose membranes with anti-DHP receptor antibodies. Positions of the 360 kDa protein and α_1 subunit of the DHP receptor (185 kDa) are indicated.


A
B
360
180
185
a
b
c
a
b
c

appearing stronger in the SL (Fig. 9B, lane a) compared with the con A-lectin column fraction (Fig. 9B, lane b).

3.4.5 Western Blotting of Cardiac and Skeletal Muscle Membranes with Anti-360 kDa Protein Antibodies:

Many proteins are common to both cardiac muscle and skeletal muscle. These proteins may be identical or may vary yet retaining a high degree of sequence homology in parts. Others only exist in one muscle type and not in another. To determine whether the 360 kDa protein was localized in skeletal muscle, crude skeletal muscle membranes were prepared, subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-360 kDa protein antibodies. No staining was observed in skeletal muscle (Fig. 10B), while staining of the 360 kDa protein was observed in control crude cardiac membranes (Fig. 10A). This indicated that the 360 kDa protein was specific to cardiac muscle.

3.5 Polyclonal Antibodies Directed Against Dystrophin

3.5.1 Production of Anti-Peptide Antibodies:

In characterizing the 360 kDa protein, other high molecular weight proteins that were associated with the sarcolemma were considered as potential candidates. One such protein was dystrophin. Dystrophin is a high molecular weight protein of Mr ~400 kDa comprising only 0.002% of total muscle protein (Hoffman, 1988). Dystrophin has been purified from skeletal muscle membranes and found to be a cytoskeletal protein (Ervasti, 1991). Dystrophin has been detected in cardiac muscle using anti-dystrophin antibodies but has not as yet been purified. Anti-dystrophin antibodies were obtained from Dr. Paul. Holland, Neurological Institute, McGill University,

Figure 10: Immunoblotting of the 360 kDa protein in cardiac and skeletal muscle sarcolemma:

Porcine cardiac SL (A) and rabbit skeletal SL (B), were prepared, subjected to 7% SDS-PAGE and transferred to nitrocellulose, all as described in "Materials and Methods". Immunoblotting of nitrocellulose membranes was carried out as described in "Materials and Methods" using anti-360 kDa protein antibodies. The position of 360 kDa protein is indicated.

360►

A B

Montréal, Québec. These antibodies were directed against the C-terminal 17 amino acids of dystrophin (seq.: SSRGRNTPGKPMREDTM) and were affinity purified on a peptide column.

3.5.2 Specificity of Anti-Peptide Antibodies for Skeletal and Cardiac Dystrophin:

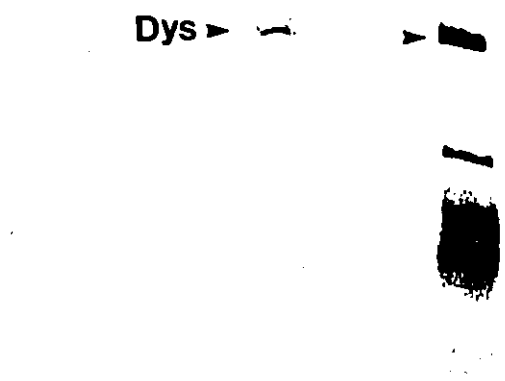
The specificity of the purified anti-peptide antibodies were tested by Western blotting against normal skeletal and cardiac muscle compared with skeletal and cardiac muscle from *mdx* mice. The *mdx* mouse is a dystrophin-deficient mouse (Hoffman, 1989) which is used as an animal model for Duchenne Muscular Dystrophy. Anti-peptide antibodies specifically recognized a 400 kDa polypeptide in normal muscle (Fig. 11A and 11C) but not in dystrophic muscle (Fig. 11B and 11D).

3.6 Immunoreactivity of the 360 kDa Protein and of Dystrophin in Subcellular Muscle Fractions

The anti-360 kDa protein antibodies and the anti-dystrophin antibodies were used to study the subcellular distribution of the 360 kDa protein and of dystrophin, respectively. Cardiac and skeletal homogenate was separated into soluble (cytosolic) fraction and crude microsomal membrane fractions as described in the "Materials and Methods". The microsomes were further separated into a light membrane fraction enriched in sarcolemmal markers and a heavy membrane fraction enriched in SR, terminal cisternae and t-tubules. Fractions were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted (Fig. 12). Similar staining was observed in all subcellular fractions with both antibodies (Fig. 12A and 12B). Both the 360 kDa protein and dystrophin were distributed in the cardiac muscle cytosolic fraction (Fig. 12A,

Figure 11: Specificity of anti-peptide antibodies for skeletal and cardiac dystrophin:

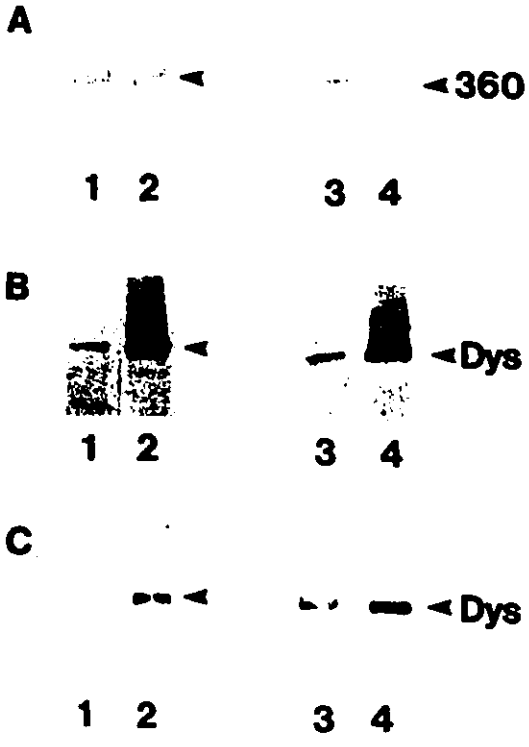
Crude preparation of rabbit skeletal (A) and porcine cardiac (C) muscle and skeletal (B) and cardiac (D) crude muscle homogenate from *mdx* mice, were separated by SDS-PAGE (7% acrylamide) and transferred to nitrocellulose, all procedures as described in "Materials and Methods". Nitrocellulose membranes were blotted with anti-peptide antibodies to dystrophin as described in "Materials and Methods". Position of dystrophin is indicated.



A B C D

Figure 12: Immunoreactivity of the 360 kDa protein and dystrophin in subcellular muscle fractions:

Subcellular fractions from cardiac (A and B) and skeletal (C) muscle were prepared, transferred to nitrocellulose and stained with either anti-360 kDa protein antibodies or anti-peptide antibodies to dystrophin, as indicated by 360 (anti-360 kDa protein antibodies) and dys (anti-dystrophin antibodies). All procedures are as described in "Materials and Methods". Lane 1, soluble fraction (50 μg); lane 2, microsomes (50 μg); lane 3, light membrane fraction (50 μg); lane 4, heavy membrane fraction (50 μg).


A
360
1 2 3 4
B
Dys
1 2 3 4
C
Dys
1 2 3 4

lane 1 and 12B, lane 1) and microsomal fraction (Fig. 12A, lane 2 and 12B, lane 2). Furthermore, both displayed similar staining in the cardiac light membrane fraction (Fig. 12A, lane 3 and 12B, lane 3). A stronger staining however was observed for dystrophin in the heavy membrane fractions (Fig. 12B, lane 4) than for the 360 kDa protein in the same fraction (Fig. 12A, lane 4). Similar studies were carried out in skeletal muscle with the anti-dystrophin antibodies for comparison. Interestingly, no staining was observed in the cytosolic fraction of skeletal muscle (Fig. 12C, lane 1). Furthermore, when comparing light versus heavy membrane fractions, skeletal muscle staining appeared approximately equal (Fig. 12C, lanes 3 and 4) whereas in cardiac muscle, a much stronger staining of dystrophin was observed in the heavy fractions. These results suggested that in cardiac muscle, dystrophin is more closely associated with the heavy membrane fraction which is known to contain transverse tubules in association with the SR rather than the light membrane fraction which is known to be enriched in surface membranes (SL) as with skeletal muscle.

3.7 Immunoreactivity of the 360 kDa Protein with Anti-Dystrophin Antibodies

Based on the similar subcellular distribution of the 360 kDa protein with dystrophin, anti-dystrophin antibodies were used in order to determine if the 360 kDa protein was actually dystrophin. Anti-dystrophin antibodies reacted with a protein of ~400 kDa in both skeletal and cardiac muscle membranes (Fig. 13A and 13B), but not with the purified 360 kDa protein derived from cardiac SL membranes (Fig. 13C). This indicated that dystrophin and the 360 kDa protein were not immunologically related.

Figure 13: Immunoreactivity of anti-dystrophin antibodies with skeletal and cardiac membranes and the 360 kDa protein:

Skeletal (A) and cardiac (B) membranes and purified 360 kDa protein (C) were separated on a 7% SDS-gel and transferred to nitrocellulose, all as described in "Materials and Methods". Nitrocellulose membranes were immunoblotted using anti-peptide antibodies to dystrophin as described in "Material and Methods". Position of dystrophin is indicated.

A B C

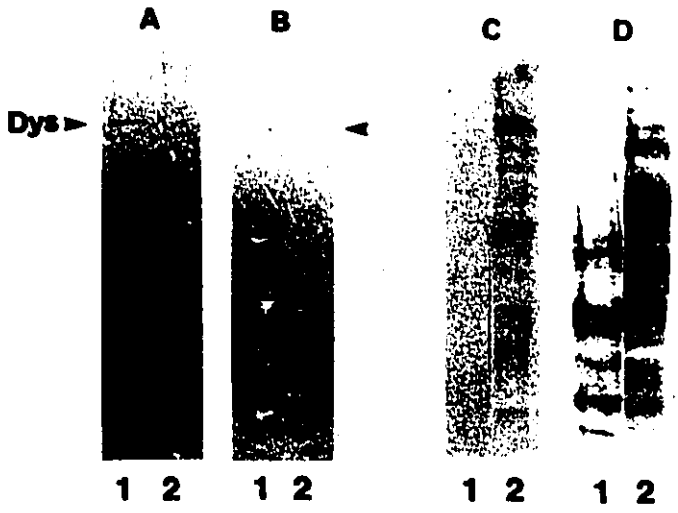
3.8 Separation of the 360 kDa Protein from Dystrophin on Lectin Columns

Previous studies on skeletal muscle dystrophin (Campbell, 1989; Yoshida, 1990; Ervasti, 1991) indicated that this protein tightly associated with sarcolemmal membrane glycoproteins and could be isolated and purified as a complex on lectin columns such as WGA-sepharose and con A-sepharose. Furthermore, a dystrophin-related protein has also been recently described (Khurana, 1990; Ohlendieck, 1991). To further investigate any relationship between the 360 kDa protein and dystrophin, the various fractions obtained during the purification of the 360 kDa protein were blotted with anti-dystrophin antibodies. When cardiac muscle membranes were solubilized with 1% NP-40 and fractionated on a con A-sepharose column, all the dystrophin appeared in the flow-through fraction (Fig. 14A, lane 1) and none in the material specifically eluted with the sugar MDM (Fig. 14A, lane 2). The 360 kDa protein however, was present in the fraction which was specifically eluted from the con A column (Fig. 9A, lane b). This further indicated that these proteins were in fact different. To prove that the column was adsorbing all con A-binding glycoproteins from cardiac membranes, the flow-through and material specifically eluted from the con A column was separated by SDS-PAGE, transferred to nitocellulose and stained with peroxidase-labelled con A. No staining was observed in the flow-through (Fig. 14C, lane 1) but strong staining was observed in the eluted material (Fig. 14C, lane 2), indicating the specific binding and elution of glycoproteins from the con A-sepharose column. When these fractions were stained with peroxidase-labelled WGA, staining was observed in both the flow-through (Fig. 14D, lane 1) and in the material specifically eluted from the column (Fig. 14D, lane 2). These results suggested that dystrophin from cardiac

Figure 14: Immunoreactivity of dystrophin in lectin column fractions:

Porcine cardiac (A, C and D) and rabbit skeletal (B) sarcolemmal membranes were prepared, solubilized with 1% NP-40, applied to a con A-sepharose column and adsorbed proteins specifically eluted with 0.25 M MDM, as described in "Materials and Methods". Both flow-through and eluted fractions were subjected to 7% SDS-PAGE, transferred to nitrocellulose and stained with anti-peptide antibodies to dystrophin (A and B), peroxidase-labelled-con A (C) and peroxidase-labelled WGA (D). For all panels: lane 1, column flow-through; lane 2, column eluate. Position of dystrophin is indicated.

Panel B demonstrates differences between cardiac and skeletal dystrophin. Dystrophin from skeletal muscle is shown to be present in the eluted fraction (data similar to Campbell, 1989) as well as in the flow-through fraction. Under identical conditions, cardiac dystrophin is present only in the flow-through fraction. This demonstrates differences in the association of dystrophin with sarcolemmal glycoproteins/proteins between the two muscles.


A
B
C
D
Dys
1 2
1 2
1 2
1 2

muscle isolated under these conditions, did not associate with con A-sensitive glycoproteins as described for skeletal muscle (Campbell, 1989).

3.9 Relationship Between the 360 kDa Protein and α_2-Macroglobulin

3.9.1 Crossreactivity of the 360 kDa Protein with α_2-Macroglobulin:

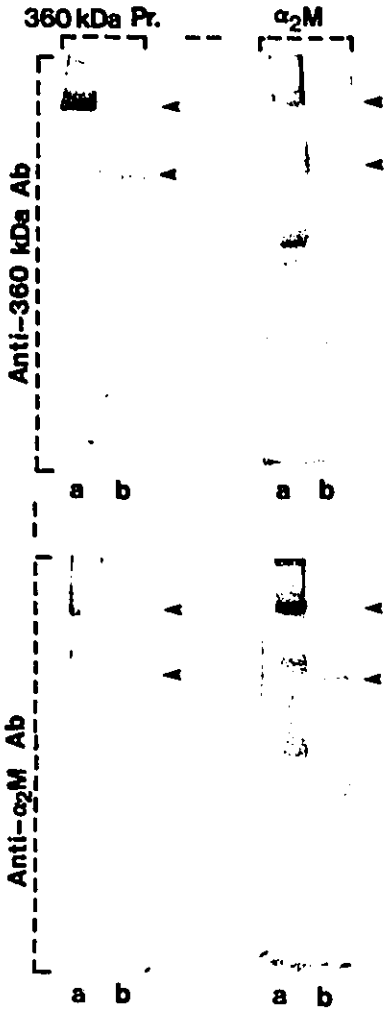
During the course of this study, the 180 kDa reduced product of the 360 kDa protein was seen to coincidently migrate with one of the molecular weight standard proteins, α_2M. Upon further investigation, α_2M was found to display a similarity in subunit composition to the 360 kDa protein. α_2M is composed of four identical subunits of Mr ~185 kDa linked in pairs by disulfide linkages and the pairs held together by noncovalent bonds. When subjected to SDS-PAGE, α_2M migrates with a Mr of 360,000 under nonreducing conditions and ~180 kDa under reducing conditions. Therefore, α_2M and α_2M antibodies were commercially obtained and the two proteins were compared immunologically as shown in Fig. 15. Anti-360 kDa protein antibodies crossreacted with α_2M and anti-α_2M antibodies crossreacted with the 360 kDa protein, both under nonreducing and reducing conditions. Crossreactivity of these antibodies indicated that both proteins were immunologically related.

3.9.2 Partial Proteolytic Cleavage of the 360 kDa Protein and α_2-Macroglobulin:

To further investigate any structural relationship between the 360 kDa protein and α_2M, the two proteins were separated on SDS-gels and stained with Coomassie Brilliant Blue. Bands corresponding to Mr 360,000 were excised and subjected to partial proteolysis with *S. aureus V8* protease. *S.*

Figure 15: Crossreactivity of the 360 kDa protein with α_2-macroglobulin:

Purified 360 kDa protein (360 kDa Pr.) and α_2M were subjected to SDS-PAGE under nonreducing (a) and reducing (10 mM DTT) (b) conditions and transferred to nitrocellulose, as described in "Material and Methods". Immunoblotting of nitrocellulose blots with anti-360 kDa protein antibodies and anti-α_2M antibodies was carried out as described in "Materials and Methods". The position of 360 kDa protein and 180 kDa reduced product are indicated by the arrows.


360 kDa Pr.
α2M
Anti-360 kDa Ab
Anti-α2M Ab
a b
a b
a b
a b

aureus V8 protease cleaves polypeptide chains on the carboxyl side of aspartate and glutamate residues. Fig. 16 shows the peptide maps of the 360 kDa protein (Fig. 16A) and α_2M (Fig. 16B) following silver staining of SDS-gels. Proteolysis generated very similar maps, which suggested that the proteins were structurally related.

3.10 Immunofluorescent Localization of the 360 kDa Protein and Dystrophin in Cardiac Muscle

3.10.1 Localization of the 360 kDa Protein in Cardiac Tissue Sections:

α_2M is a protease inhibitor which to-date, has also been detected in the liver and various body fluids such as plasma, synovial, lung lavage and ascites fluids (Van Leuven, 1982). Biochemical and immunological studies conducted with the 360 kDa protein have indicated that the 360 kDa protein is very closely related to α_2M. In view of the fact that α_2M was a protease inhibitor found in plasma, we were concerned as to whether α_2M from the blood was contaminating the SL membrane preparations. In the preparation of SL membranes, porcine hearts were extensively washed with buffer to remove blood from the heart. However, despite the precautions taken, it was possible that α_2M was contaminating the preparation. In order to determine the localization of the 360 kDa protein, immunofluorescence localization was carried out in cardiac tissue sections as shown in Fig. 17. Longitudinal and transverse tissue sections from porcine ventricular muscle stained with anti-α_2M antibodies (non-purified and purified) followed by FITC-IgG secondary antibody Fig. 17D and 17E, respectively, showed fluorescence staining along the SL membrane. Controls (Fig. 17B and 17C) showed no fluorescent staining.

Figure 16: One-dimensional peptide maps of the 360 kDa protein and α_2-macroglobulin:

Con A-purified 360 kDa protein, 50 µg (A) and pure α_2M, 50 µg (B) were separated on 7% SDS-PAGE and stained with Coomassie Brilliant Blue. Bands corresponding to a molecular weight of 360 kDa were excised, digested with *S. aureus V8* protease, separated on 15 % SDS-PAGE and stained with silver, all as described in "Materials and Methods". Protease band is indicated.

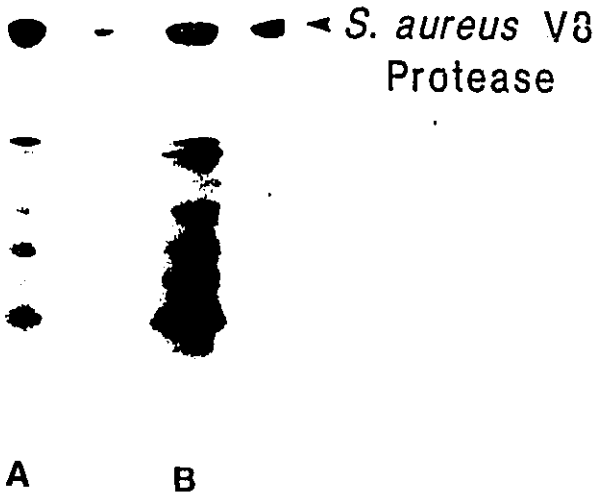
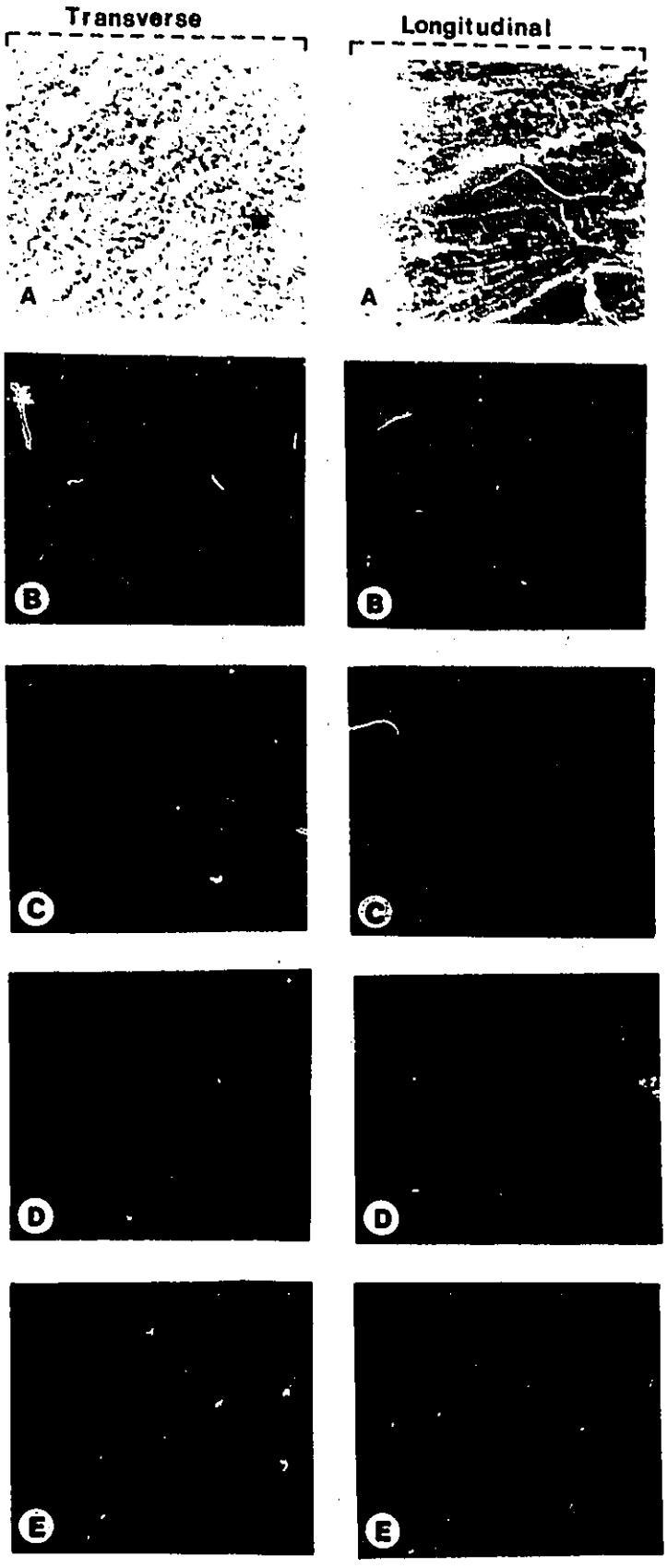

S. aureus V8
Protease
A
B

Figure 17: Indirect immunofluorescent localization of the 360 kDa protein in cardiac tissue sections:

Longitudinal and transverse tissue sections from porcine ventricular muscle (8 μm) were prepared as described in "Materials and Methods". Sections were stained with primary antibody followed by fluorescein-conjugated anti-rabbit IgG secondary antibody, all as described in "Materials and Methods". (A) phase contrast of tissue section; (B) section incubated with secondary antibody alone; (C) section incubated with α_2M antibodies absorbed with pure α_2M; (D) section incubated with α_2M antibodies; (E) section incubated with purified α_2M antibodies. Magnification 40x for phase contrast; 63.1x for fluorescence.

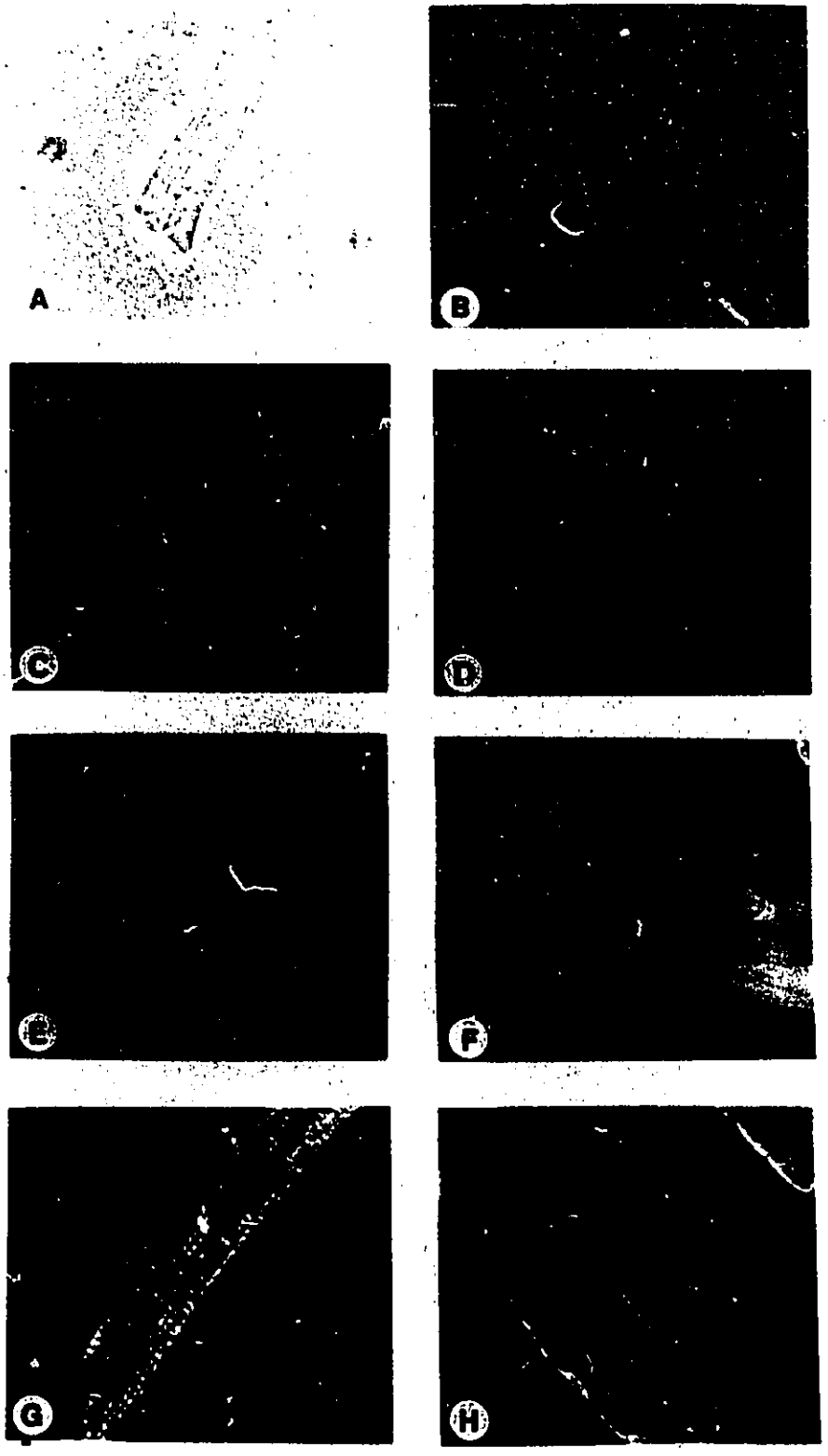

Transverse
Longitudinal
A
A
B
B
C
C
D
D
E
E

3.10.2 Localization of the 360 kDa Protein and Dystrophin in Cardiomyocytes:

The localization of the 360 kDa protein was further studied in the isolated cardiomyoctye. Cardiomyocytes were obtained from the laboratory of Dr. M. Désilets, Department of Physiology, University of Ottawa. In the preparation of cardiomyoctyes, the heart was pumped several times with solution in order to press blood out of the heart. The heart was then mounted on a Langendorff apparatus and the aorta perfused retrogradely with buffer to flush out the remaining blood. Isolated cells were not treated with serum as with some isolation procedures. Therefore, the cells were free of contamination of α_2M from the blood. Anti-α_2M antibodies were used in localization studies in place of the anti-360 kDa protein antibodies for various reasons. Firstly, anti-α_2M antibodies (Sigma) were affinity purified representing the IgG fraction of serum, whereas anti-360 kDa protein antibodies remained to be purified. Purification of these antibodies would have been a long process because of the amount of protein needed to be purified in order to make an immunoaffinity column for antibody purification. Secondly, the biochemical similarities and immunological crossreactivities between these two proteins were established, as noted above. Localization of the 360 kDa protein is shown in Fig. 18. Fig. 18A represents a freshly isolated rabbit ventricular myocyte showing the typical cross-striations and intercalated discs characteristic of cardiac ventricular muscle. Fig. 18B-18D represent control cells incubated with secondary antibody alone under permeabilized and non-permeabilized conditions and cells incubated with α_2M antibodies absorbed with α_2M, as described in "Materials and Methods". No fluorescence was observed in the control cells. Similarly, no fluorescence was observed in cells incubated with anti-α_2M antibodies under non-permeabilized conditions (Fig. 18E). However under permeabilized conditions (0.2% Triton X-100, a mild nonionic detergent), fluorescence was

Figure 18: Indirect immunofluorescent localization of the 360 kDa protein and dystrophin in cardiomyocytes:

Rabbit ventricular cardiomyocytes were stained with primary antibody followed by incubation with fluorescein-conjugated anti-rabbit IgG secondary antibody, as described in "Materials and Methods." (A) phase contrast of cardiomyoctye; (B) non-permeabilized cardiomyocyte incubated with secondary antibody alone; (C) permeabilized cardiomyocyte incubated with secondary antibody alone; (D) permeabilized cardiomyocyte incubated with α_2M antibodies absorbed with α_2M; (E) non-permeabilized cardiomyocyte incubated with α_2M antibodies; (F) permeabilized cardiomyocyte incubated with α_2M antibodies; (G and H) permeabilized cardiomyocyte incubated with anti-peptide antibodies to dystrophin. Magnification 40x for phase contrast; 63.1x for fluorescence.


A
B
C
D
E
F
G
H

observed along the interior of the cell, close to the cell membrane as well as throughout the cytosol (Fig. 18F). This indicated that the 360 kDa protein was from the muscle and not α_2M from the blood.

Since the subcellular distribution of the 360 kDa protein and of dystrophin was similar (Fig. 12), localization of dystrophin was carried out in cardiomyocytes in order to further compare the distribution. Anti-dystrophin antibodies gave a consistent and strong punctate staining pattern throughout the permeabilized cardiomyocytes and also along the inner face of the SL (Fig. 18G and 18H). No reactivity was observed with FITC-IgG secondary antibody control (Fig. 18B). The distances between fluorescent dots in the dystrophin stained cells were measured and ranged between 2.2-2.4 μm. The sarcomere length or distance between the Z-lines in resting cardiac muscle ranges between 1.5-2.5 μm (Page, 1974). In cardiac muscle, t-tubules invaginate predominately at the Z-line of the sarcomere, whereas in skeletal muscle, t-tubules invaginate the cell at the level of the A-I band of the sarcomere (Adams, 1980). This suggests that dystrophin in cardiac muscle is predominately localized to either the Z-line or t-tubules. In light of the subcellular fractionation sudies, it is proposed dystrophin is localized to the t-tubules.

4.0 DISCUSSION

The voltage-sensitive Ca^{2+} channel (DHP receptor) plays a pivotal role in excitation-contraction coupling. Understanding its structure is vital in understanding its function. The subunits comprising the DHP receptor from skeletal muscle have been purified, characterized and cloned. As a result, some of the specific regions critical to the channel's activity have been identified. Purification of the Ca^{2+} channel from cardiac muscle however has been hampered due to its low abundance and high susceptibility to proteolytic attack.

The DHP receptor from cardiac muscle has been localized to the cell membrane and partially purified by this laboratory (Tuana, 1987). During purification, a high molecular weight protein of Mr 360,000 was found to co-purify with the DHP receptor. This observation was of particular interest as the 360 kDa protein co-purified with the DHP receptor through three different purification steps. Therefore, the possible relationship of these two proteins was investigated.

A simplified and efficient protocol was developed for the purification of the 360 kDa protein. During the purification, the DHP receptor did not remain associated with the 360 kDa protein. Furthermore, my studies indicated that there was no immunological relationship between the two proteins since antibodies directed against the DHP receptor did not recognize a protein corresponding to its molecular weight (185 kDa) in the purified 360 kDa fraction. Additionally, these antibodies did not crossreact with the 360 kDa protein. Antibodies raised against the 360 kDa protein did not crossreact with the DHP receptor either. These results demonstrated that the 360 kDa protein and the DHP receptor were neither associated nor related proteins.

The possibility that the 360 kDa protein was dystrophin was investigated since dystrophin is a high molecular weight protein localized to the SL. In cardiac and skeletal muscle, dystrophin has been reported to be uniformly distributed along the inner surface of the plasma membrane (Arahata, 1988; Bonilla, 1988; Zubrzycka-Gaarn, 1988), in tight association with sarcolemmal glycoproteins (Campbell, 1989; Yoshida, 1990; Ervasti, 1991). Absence of dystrophin is the primary biochemical defect in patients with Duchenne muscular dystrophy (Hoffman, 1987) and leads to segmental necrosis of their skeletal myofibers. Although present in similar amounts in normal cardiac and skeletal muscle, the absence of dystrophin from cardiac muscle has less severe effects on the survival of cardiac cells (Engel, 1986). Subcellular fractionation studies demonstrated that both the 360 kDa protein and dystrophin were distributed in the cytoplasm (soluble fraction) and various membrane fractions from cardiac muscle. This indicated a possible relationship between these proteins. However, anti-dystrophin antibodies did not crossreact with the 360 kDa protein. Furthermore, dystrophin was not detected in the partially pure 360 kDa protein fraction obtained from the con A-lectin column. While the 360 kDa protein was present in the fraction specifically eluted from the lectin column, dystrophin was instead present in the column flow-through. This indicated that the 360 kDa protein was not dystrophin. Interestingly, this study showed that cardiac dystrophin was associated with the SL but not through an association with con A-sensitive glycoproteins. This was contrary to that observed with skeletal muscle in which dystrophin was shown to be specifically eluted and isolated from lectin-afffinity columns (Campbell, 1989; Yoshida, 1990, Ervasti, 1991). Further to this, subcellular fractionation studies showed a difference in the distribution of dystrophin between the two muscles. Cardiac dystrophin was found to exist in both soluble and membrane-bound forms, whereas skeletal

dystrophin was found only in membrane bound forms (Campbell, 1989). Human dystrophin proteins have been shown to be present in the cytoplasm and sarcolemma of myofibres when human dystrophin expression plasmids were injected intramuscularly into dystrophin-deficient *mdx* mice (Acsadi, 1991). Additionally, three alternatively spliced isoforms of dystrophin, differing at the C-terminus have been detected by PCR amplification of both cardiac and skeletal muscle cDNA (Feener, 1989). Differences in the relative abundance of each isoform between skeletal and cardiac muscle could therefore be responsible for the properties of cardiac dystrophin detected in the present study. The anti-dystrophin antibodies were raised against a peptide at the C-terminal end, thus these results do not unequivocally rule out a relationship between the 360 kDa protein and dystrophin.

The localization of the 360 kDa protein was carried out in cardiac tissue sections and staining appeared along the SL membrane. Dystrophin has also been shown to stain the SL in cardiac tissue sections (Bonilla, 1988). Based on this and the similar subcellular distribution of these proteins, the localization of the 360 kDa protein and dystrophin was investigated in cardiomyocytes. Interestingly, both proteins displayed a similar pattern of staining. Staining appeared along the inner SL and throughout the cytoplasm of the cardiomyocyte. Particularly striking was the distinct punctate staining pattern for dystrophin seen throughout the cytosol, approximating to the level of the t-tubules/Z-line. While dystrophin has been found in t-tubular fractions of skeletal muscle (Knudson, 1988; Salviati, 1989), the bulk of dystrophin appears sarcolemmal on immunohistochemical staining (Arahata, 1988; Bonilla, 1988; Zubryzycka-Gaarn, 1988).

In view of the similar subcellular distribution and localization of the 360 kDa protein and dystrophin, further biochemical and immunological studies

were conducted with the 360 kDa protein in order to determine its structure and possible function. The 360 kDa protein was found to be a dimeric glycoprotein composed of monomers of Mr 180,000 held together by disulfide bonds. Additionally, anti-360 kDa proteins crossreacted with α_2M and anti-α_2M antibodies crossreacted with the 360 kDa protein. Furthermore, similar peptide maps were generated with the 360 kDa protein and α_2M. These data strongly suggest that the 360 kDa protein and α_2M are related. α_2M is a high molecular weight glycoprotein (Mr 725,000) composed of four monomers of Mr 180,000. Two monomers are held together by disulfide bonds and the two pairs of monomers held together by noncovalent bonds. α_2M therefore migrates to Mr 360,000 in SDS-gels as does the 360 kDa protein.

α_2M is a protease inhibitor found in the plasma of vertebrates and invertebrates (Starkey, 1982; Quigley, 1983; Spycher, 1987). It has the unique property of binding and inhibiting the majority of endopeptidases (thio, carboxyl, serine and metallo proteases), regardless of their sensitivity or catalytic mechanism. α_2M inhibits the proteolytic activity of these proteases via a trapping mechanism. This involves the cleavage of a peptide bond in a region called the "bait region" of the α_2M. This results in a conformational change in α_2M such that that protease becomes physically entrapped within the molecule. The α_2M-proteinase complex is then cleared by receptor-mediated-endocytosis, primarily by receptors on hepatocytes (Van Leuven, 1982). Receptor binding and recognition has been shown to be Ca^{2+} dependent (Moestrup, 1990). The half-life of the complex is 2-5 min. α_2M is synthesized in the liver and is then transported to the blood where its acts. However in the past few years, other tissues have been shown to synthesize α_2M or α_2M-like proteins (Nagase, 1982; Gliemann, 1983; Van Leuven, 1986; Gaddy-Kurten, 1989; Kirshner, 1989; Shi, 1990). The overall physiological function of α_2M in

these tissues is not known, although its function as a protease inhibitor has been proposed.

This study has demonstrated the presence of an α_2M-like protein in cardiac muscle. The α_2M-like protein was found to be present in cardiac muscle, but not in skeletal muscle. Immunofluorescent localization studies showed that this protein was located in the cell membrane as well as throughout the cytosol of cardiomyocytes. This was consistent with the subcellular fractionation studies. The function of the α_2M-like protein is also speculated to be a protease inhibitor in cardiac muscle. Several proteases exist both in the membrane and cytosol, continuously degrading proteins (Wildenthal, 1980, Bond, 1987). The significance of this degradation remains uncertain, however it is believed that turnover of proteins may facilitate the adaptation of the organism to its environment. For instance, exogenous (mutagens) or endogenous (biosynthetic) factors can be removed quickly by the action of these proteases. Additionally, adaptation to poor environments may require degradation of proteins to provide energy and sufficient amino acid precursors for the sysnthesis of those proteins which are required. The presence of these proteases necessitates the presence of protease inhibitors in order to control proteolysis of proteins within the cell, thereby producing a homeostatic environment. The α_2M-like protein may therefore be one such inhibitor.

In view of the role of α_2M as an endogenous protease inhibitor, its co-purification with the DHP receptor and its subcellular distribution with dystrophin, it is suggestive that the α_2M-like protein may be protecting these proteins from proteolytic degradation. This, along with the specificity of the α_2M-like protein for cardiac muscle, is extremely interesting especially when considering excitation-contraction-coupling in the heart and the role of

extracellular Ca^{2+}. Several Ca^{2+}-activated proteases are localized in the cytosol and membranes of muscle cells. During contraction, the increase in $[Ca^{2+}]$ activates these proteases. These proteases have been reported to fall into two classes on the basis of their requirement for millimolar or micromolar concentration of Ca^{2+} (Mellengren, 1980; Mellengren, 1982). Ca^{2+}-activated proteases have been shown to degrade myofibrillar proteins *in vitro*. Furthermore, Reddy *et al* (1982) demonstrated the removal of Z-lines and α-actinin from isolated myofibrils by Ca^{2+}-activated neutral proteases and the stringent requirement for Ca^{2+} as opposed to other divalent cations. Interestingly, these proteases have been found to be markedly elevated in muscle of patients with Duchenne muscular dystrophy (Kar, 1980). α_2M with its broad specificity of action would be capable of inhibiting these thiol proteases and therefore the excessive degradation of myofibrillar proteins as well as proteins such as dystrophin and ion channels.

Endopeptidases such as cathepsin B and cathepsin H which are present in muscle, are known to degrade a wide variety of proteins including myofibrillar proteins (Barrett, 1973a). Furthermore, these proteases have been shown to interact with α_2M (Barrett, 1973b; Mason, 1989). This further supports the hypothesis that the α_2M-like protein may be acting in the myocardium to prevent excessive degradation of proteins during excitation-contraction coupling.

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