CHARACTERIZATION OF HEART SARCOLEMMAL Ca^{2+}/Mg^{2+} ATPase

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CHARACTERIZATION OF HEART SARCOLEMMAL Ca^{2+}/Mg^{2+} ATPase

BY

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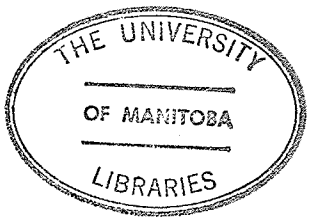

To my father and late mother with great love and affection

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ABSTRACT

In a series of experiments, rat heart sarcolemmal, mitochondrial and microsomal fractions were isolated and their ATP hydrolyzing activities compared. Several divalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+} and Sr^{2+} were able to stimulate ATP hydrolysis by rat heart sarcolemmal, mitochondrial and microsomal fractions, however, the order of their potency was different for each fraction. Maximal activities in the presence of Ca^{2+} and Mg^{2+} were obtained at 4 - 8 mM concentrations under the experimental conditions employed in this study, however, the mitochondrial ATPase activity was higher than that of sarcolemma but lower than that of microsomes. The pH optima for mitochondrial and microsomal ATPase varied between 8.0 - 8.5 whereas that for sarcolemma was observed at 7.5 - 8.0. Unlike sarcolemma and microsomes, mitochondrial Ca^{2+} or Mg^{2+} ATPase were stimulated by DNP and inhibited by sodium azide; sarcolemmal Mg^{2+} ATPase was slightly inhibited by sodium azide. Although NaF, iodoacetate and ruthenium red inhibited sarcolemmal, mitochondrial and microsomal ATPases, some differences in sensitivities to these agents were apparent. Lanthanum in low concentrations significantly inhibited sarcolemmal Ca^{2+} or Mg^{2+} ATPase only. Ca^{2+} ATPase and Mg^{2+} ATPase activities of sarcolemma, microsomes, and mitochondria were decreased by PCMB except that this agent had a biphasic effect on sarcolemmal Mg^{2+} ATPase and decreased the microsomal enzyme activities to a greater extent than those of the mitochondria. Iodoacetamide stimulated mitochondrial Ca^{2+} ATPase and sarcolemmal Mg^{2+} ATPase but decreased microsomal Mg^{2+} ATPase activity. Carbodiimide inhibited microsomal Ca^{2+} and Mg^{2+} ATPase activity. On the other hand, sarcolemmal Ca^{2+} ATPase and mitochondrial

Mg^{2+} ATPase activities were depressed and stimulated by maleic anhydride respectively. These results indicate some similarities and differences among Ca^{2+}/Mg^{2+} ATPase systems of cardiac sarcolemma, mitochondria and sarcoplasmic reticulum.

In order to gain further information concerning the properties of the heart sarcolemmal ATPase systems, the ATP hydrolyzing activities of the membrane in the presence of Ca^{2+} or Mg^{2+} were tested under different conditions. It was found that the Ca^{2+} ATPase activity was higher than the Mg^{2+} ATPase activity; however, both of these enzyme systems had two sites for ATP. The K_m value for the low affinity site for Ca^{2+} ATPase and Mg^{2+} ATPase was about 318 μM whereas those for the high affinity sites were about 81 and 106 μM respectively. The V_{max} values for the low affinity sites for Ca^{2+} ATPase and Mg^{2+} ATPase were 33 and 25 μmoles Pi/mg/hr respectively whereas that for the high affinity sites varied between 15.1 - 15.6 μmoles Pi/mg/hr. The K_a values for Ca^{2+} ATPase and Mg^{2+} ATPase were 0.57 - 0.68 and 0.71 - 0.88 mM respectively. Sarcolemma hydrolyzed nucleotides other than ATP but the order of potency in the presence of Ca^{2+} was different from that in the presence of Mg^{2+}. Storage of sarcolemma at 0 - 2°C increased ATPase activities whereas the products of ATP hydrolysis such as ADP and Pi inhibited the enzyme activity; however, the extents of changes in the presence of Ca^{2+} were different from those in the presence of Mg^{2+}. Freezing and thawing as well as preheating the membrane decreased the Ca^{2+} ATPase and Mg^{2+} ATPase activities. Different monovalent cations such as Na^+, K^+, NH_4^+, Li^+ and Cs^+ had no effect whereas divalent cations such as Co^{2+}, Mn^{2+}, Ni^{2+} and Cu^{2+}, but not Ba^{2+} and Sr^{2+}, inhibited the sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase activities. Cyclic AMP - protein kinase increased

the Ca^{2+} ATPase and Mg^{2+} ATPase activities while cyclic AMP or epinephrine were not effective. These results indicate that there are some differences between Ca^{2+} ATPase and Mg^{2+} ATPase of the heart sarcolemma and support the view that this enzyme complex may be involved in some manner in a process associated with Ca^{2+} influx.

Treatment of heart sarcolemma with phospholipases A, C and D produced varying degrees of morphological changes and decreased calcium binding ability. The Mg^{2+} ATPase activity was decreased upon treating heart sarcolemma with phospholipases, A, C and D; phospholipase A produced the most dramatic effect. The reduction in Mg^{2+} ATPase activity by each phospholipase treatment was associated with a decrease in the V_{max} value without any changes in the K_a value. The decreased Mg^{2+} ATPase in the phospholipase treated preparations was not found to be due to release of saturated or unsaturated fatty acids in the medium and was not restored upon reconstitution of these membranes by the addition of synthetic phospholipids such as lecithin, lysolecithin or phosphatidic acid. In contrast to the Mg^{2+} ATPase, the sarcolemmal Ca^{2+} ATPase was affected only slightly by phospholipase treatments. The greater sensitivity of Mg^{2+} ATPase to phospholipase treatments was also apparent when deoxycholate-treated preparations were employed. These results indicate that more glycerophospholipids are required for the sarcolemmal Mg^{2+} ATPase activity than that for the Ca^{2+} ATPase activity and the phospholipids associated with Mg^{2+} ATPase are predominantly exposed at the outer surface of the membrane.

Trypsin was found to stimulate the heart sarcolemmal Ca^{2+} ATPase without affecting the Mg^{2+} ATPase activity. The increase in Ca^{2+} ATPase activity was observed to be due to a decrease in the K_a value from 0.59

to 0.45 mM and an increase in the V_{max} value from 37 to 69 µmoles Pi/mg/hr. On the other hand, the Mg^{2+} ATPase activity was not appreciably affected by trypsin. In membrane preparations treated with detergents, such as deoxycholate and lubrol, trypsin was found to decrease the Mg^{2+} ATPase activity whereas the Ca^{2+} ATPase activity was increased. Trypsin treatment released proteins in the supernatant which showed ATP hydrolysis in the presence of Ca^{2+} but not Mg^{2+}. The K_a and V_{max} values for the supernatant Ca^{2+} ATPase were 0.35 mM and 54 µmoles Pi/mg/hr respectively. The release of Ca^{2+} ATPase in the supernatant upon trypsin treatment suggests the peripheral nature of this enzyme in heart sarcolemma.

Digestion of sarcolemma with tryspin produced dramatic changes in the membrane structure as well as electrophoretic pattern. The specific activities of Ca^{2+} ATPase and Mg^{2+} ATPase in the trypsin-treated preparations increased by 2.5 and 3 fold respectively whereas the membrane protein decreased by about 60%. Although increases in both Ca^{2+} ATPase and Mg^{2+} ATPase activities in trypsin-treated preparations with millimolar concentrations of cations were associated with increases in their V_{max} values without any changes in their K_a values, the activation of Mg^{2+} ATPase, unlike Ca^{2+} ATPase, in micromolar cationic concentrations appeared to be allosteric in nature. The V_{max} values, but not the K_m values, for low and high affinity sites for ATP in trypsin-treated membranes for both Ca^{2+} ATPase and Mg^{2+} ATPase were higher than the control; however, the K_m value for the high affinity sites for Ca^{2+} ATPase was lower than that for the Mg^{2+} ATPase. Although pH optima for Ca^{2+} ATPase and Mg^{2+} ATPase in trypsin-treated membranes were similar to the control values, Ca^{2+} ATPase in the trypsin-treated preparations was inhibited to a lesser extent by Mg^{2+} in comparison to the control whereas the magnitudes of inhibition in Mg^{2+} ATPase activities by Ca^{2+}

in both preparations were similar. These results further suggest that the properties of heart sarcolemmal Ca^{2+} ATPase are different from those of the Mg^{2+} ATPase.

I. INTRODUCTION AND STATEMENT OF THE PROBLEM

Heart sarcolemma, by virtue of its ability to control the transport of different cations and substrates, is considered to play a crucial role in regulating myocardial function and metabolism. Events leading to activation of the cardiac contractile apparatus upon excitation are believed to be initiated by a release of calcium from superficial sites in the sarcolemma, whereas relaxation of the myocardium is considered to be associated with lowering of the intracellular concentration of calcium , partly by removing calcium through the sarcolemmal membrane. Recently, several investigators have reported the isolation of heart sarcolemma containing high specific activities of important enzymes such as $Na^+ - K^+$ ATPase, adenylate cyclase and Ca^{2+}/Mg^{2+} ATPase. A great deal of work has been done concerning the properties as well as the involvement of adenylate cyclase and $Na^+ - K^+$ ATPase in cardiac function and metabolism; however, relatively little is known about Ca^{2+}/Mg^{2+} ATPase of the sarcolemma.

It is generally believed that influx of calcium in the cardiac cell during an action potential is a passive phenomenon, as calcium movements take place along the concentration gradient. Furthermore, it is assumed that depolarization of the myocardial cell leads to an increase in membrane permeability and Ca^{2+} ions move in through the slow channels in the sarcolemma. However, the molecular events associated with opening of calcium channels upon depolarization are not understood. Recently, some evidence has been presented to indicate that calcium influx in myocardium is controlled metabolically and it has been suggested that opening of calcium channels is an ATP dependent mechanism. Thus it is

tempting to consider an ATP hydrolyzing system in sarcolemma which could become activated upon depolarization and provide energy for opening calcium channels. This role can be ascribed to a Ca^{2+} ATPase, which has been shown to be present in the cardiac sarcolemma. A marked increase in the Ca^{2+} ATPase activity upon stimulating heart sarcolemma electrically, under in vitro conditions, has been observed in our laboratory. It has also been shown that positive inotropic agents such as norepinephrine, theophylline and histamine appear to act by elevating cyclic AMP levels and increasing the number of Ca^{2+} channels available for voltage activation. Furthermore, divalent cations which depress calcium influx into the cardiac muscles were found to inhibit sarcolemmal Ca^{2+} ATPase. Thus, it may be reasonable to postulate that sarcolemmal Ca^{2+} ATPase is involved in opening the calcium channels by providing energy due to ATP hydrolysis.

In addition to the presence of Ca^{2+} ATPase, heart sarcolemma has been shown to contain Mg^{2+} ATPase activity. However, it is not known, whether ATP hydrolysis in the presence of Ca^{2+} or Mg^{2+} is due to the presence of the same enzyme complex. In this regard it should be pointed out that very little is known about the movements of Mg^{2+} across the heart sarcolemma in spite of the fact that this cation is known to play an important role in myocardial function and metabolism. Hence the possibility that Mg^{2+} ATPase might be involved in the transport of magnesium can not be ignored. Furthermore, the significance of the sarcolemmal Mg^{2+} ATPase and Ca^{2+} ATPase in heart function can be appreciated from the fact that the activities of these enzymes were decreased in different types of failing hearts. It is therefore planned to undertake a detailed investigation concerning the properties of the heart

sarcolemmal Ca^{2+}/Mg^{2+} ATPases. Since other membrane systems such as sarcoplasmic reticulum and mitochondria are also known to contain an ATPase complex, which is stimulated by divalent cations, it is the purpose of this study to compare the properties of Ca^{2+}/Mg^{2+} ATPases of heart sarcolemma, mitochondria and sarcoplasmic reticulum.

Although membrane structural integrity, with respect to phospholipids and proteins, has been demonstrated to play an important role in determining the activities of membrane - bound enzymes such as Na^{+} - K^{+} ATPase, adenylate cyclase and Ca^{2+} - stimulated Mg^{2+} dependent ATPase, no such information regarding the sarcolemmal Ca^{2+}/Mg^{2+} ATPase is available in the literature. It is therefore planned to study changes in the Ca^{2+}/Mg^{2+} ATPase activities upon treatments of heart sarcolemma with phospholipase A, C and D as well as trypsin. All these membrane disrupting agents will be used in different concentrations and the treatments will be carried out for different intervals. The results will be analyzed in terms of K_m and V_{max} values to gain insight into the mechanisms responsible for alterations of the enzyme activities.

II. REVIEW OF THE LITERATURE

The importance of various cellular components such as sarcolemma, mitochondria, sarcoplasmic reticulum and myofibrils in cardiac function and metabolism is being recognized. The participation of these organelles in contractile events is based primarily on their abilities to regulate the intracellular concentration of free calcium, which has been shown to play a key role in the cardiac excitation - contraction coupling and relaxation processes (1 - 4). Over the past decade a considerable amount of work has been conducted concerning the roles of mitochondria and sarcoplasmic reticulum in heart function and metabolism. Both sarcoplasmic reticulum and mitochondria are known to accumulate calcium by energy dependent mechanisms. In addition, mitochondria generate a major portion of the cellular ATP through the process of oxidative phosphorylation. Likewise, myofibrils by virtue of their calcium binding and ATP hydrolyzing abilities provide energy for the contractile work. On the other hand, it is only recently that the heart sarcolemmal preparation has become available, in a relatively pure form, and its properties are being investigated in several laboratories for gaining information concerning its involvement in heart function.

A. Calcium Movements at Sarcolemma:

The role of calcium as a central cation in the excitation - contraction coupling and relaxation processes is well established (5 - 10). Accordingly, it is believed that calcium is a link between the excitatory event taking place at cell membrane and the contraction process that occurs at the sarcomere level. Electrical depolarization initiated by the pace-maker is propagated along the sarcolemma and it is considered to enter the cell through a transverse tubular system. This process is associated

with an influx of calcium from the extracellular space as well as a release of calcium from superficial sites in the sarcolemma. In addition, there is a release of calcium from the intracellular stores such as sarcoplasmic reticulum and possibly mitochondria through direct and indirect mechanisms. All these sources of calcium contribute in raising the intracellular concentration of free calcium from about 10^{-7} M to 10^{-6} - 10^{-5} M. This calcium binds to troponin and relieves the inhibition exerted by the troponin-tropomyosin system upon actin and myosin whereas hydrolysis of ATP due to stimulation of actomyosin ATPase provides energy for contraction of the myofibrils. The cytoplasmic concentration of free calcium is then lowered by a variety of mechanisms involving the sarcoplasmic reticulum, sarcolemma and possibly mitochondria and this event is believed to be associated with relaxation of the myofibrils. Thus the cardiac contraction and relaxation cycle is generally viewed as a reflection of raising and lowering the intracellular concentration of free calcium.

The involvement of extracellular calcium in coupling excitation with contraction in cardiac muscle has been implicated repeatedly. Electrophysiological studies have provided evidence for a slow inward current of the cardiac action potential to be due to calcium (11 - 17). The magnitude of this current during the plateau phase of the action potential was unaffected by external sodium ion concentration or tetrodotoxin, a specific antagonist of sodium influx. However it was dependent upon the external calcium concentration and was sensitive to calcium antagonists such as verapamil and its methoxy derivative, D600 (18). Various cations such as Ni^{2+}, Co^{2+}, Mn^{2+} and La^{3+} were also found to depress contractile force by inhibiting the slow inward calcium current

(19, 20). However, other studies have failed to demonstrate a clear relationship between changes in the slow inward calcium current and contractile activity under experimental conditions such as the "staircase" phenomenon (13, 21, 22) or Na^+ - free perfusion (9, 17). This raises the question whether the slow inward calcium current as determined by the electrophysiological methods represents the total amount of calcium which enters the myocardial cell upon depolarization. It should also be mentioned here that raising the internal concentration of calcium may increase potassium conductance (23 - 25). Thus, the superimposition of a potassium outward current could largely mask the calcium inward current and make it appear much less than it otherwise might be (26). The definitive answer in this regard would require careful measurements of calcium influx per beat by using some direct approaches.

On the basis of the total charge due to the calcium current it has been calculated that only 5 - 10 µmol calcium influx/kg of heart weight occurs during depolarization (14, 16). However, biochemical studies concerning measurements of myofibrillar calcium binding, ATPase activity and isometric tension at different concentrations of calcium revealed that 80 - 100 µmol of calcium/kg of heart weight were necessary for full tension development by the myocardium (27, 28). From such experiments, it is clear that the calcium influx as measured by voltage clamp techniques is not sufficient to fully activate the contractile apparatus upon depolarization. This point has been emphasized by Bassingthwaighte and Reuter (29) who presented evidence to support the view that additional calcium must be released from some intracellular sites for full cardiac contraction. This however does not undermine the essential role played by extracellular calcium because La^{3+}, which does

not penetrate the cell membrane, was found to uncouple excitation from contraction (30, 31). Furthermore, some calcium could be conceived to enter the myocardial cell by mechanisms other than that detectable by electrophysiological methods.

Since myocardial cells such as the atrial and purkinje cells (32) as well as the ventricular cells of embryonic or neonatal chickens have no transverse tubules (33), it appears that this membraneous system is not essential for the rapid propagation of the wave of excitation throughout these cells or the coupling of excitation to contraction. It should also be noted that the time between excitation and the onset of contraction of cardiac muscle is about 20 msec. Thus it is not necessary that the wave of excitation travelling along the transverse tubules releases calcium from the sarcoplasmic reticulum at the level of each sarcomere. On the other hand, current evidence points to the sarcolemmal basement membrane as the source of coupling calcium in the cardiac fiber (34). Accordingly, the calcium which is presented to the area of the contractile filament is released from the superficial cell membrane sites and travels inward to the area of the sarcomeres by a simple process of diffusion. Calcium ion diffuses into the myoplasm at a rate of 1 μm/msec and would thus take 5 msec to reach the contractile unit of the cardiac cell with an average diameter of 10 μM. This would explain the time lag between excitation and the onset of contraction. However it should be pointed out that the exact localization of the superficial sites in sarcolemma is not defined. It may represent calcium bound at the basement membrane, plasma membrane or subsarcolemmal cisternae. The possibility of the transverse tubules as a site for the superficial calcium store in some cardiac cells can not be ruled out at present.

It is considered that the superficial sites in heart sarcolemma are in equilibrium with calcium in the interstitial space; however, it is difficult to determine the amounts of calcium released from the superficial sites and that entering from the extracellular spaces during depolarization. In this regard it should also be noted that the voltage clamp methods do not detect the nonelectrogenic movement of calcium into the myocardium as well as that released from the superficial sites in sarcolemma upon depolarization. Niedergerke (35) has already claimed that external calcium enters the myocardial cell through a carrier system. The existence of such a nonelectrogenic carrier system, which moves calcium inward and sodium outward, has been suggested by some investigators (36 - 38). A calcium-potassium exchange carrier has also been proposed for the myocardium (39). Thus it appears that calcium enters the myocardial cell from the extracellular spaces and superficial sites in sarcolemma upon depolarization via electrogenic and nonelectrogenic (carrier) mechanisms.

B. Sarcolemmal Structure and Composition:

Although considerable information is available on the electrical behaviour of the heart cell membrane, very little is known about its molecular composition and structure. This is mainly because of the difficulties involved in obtaining sufficient material uncontaminated by intracellular organelles for biochemical analysis. The sarcolemma of the cardiac muscle has two components: (a) a thin electron dense plasma membrane (approximately 90 A° thick) and (b) a much thicker layer, basement membrane which coats plasma membrane. The sarcolemma with its basement membrane invaginates at regular intervals into the myocardial cells forming the transverse tubular system which comes into close

apposition with the lateral sacs of the sarcoplasmic reticulum, terminal cisternae. The basement membrane is composed of an inner layer of glycoprotein (approximately 200 A° thick) called the cell coat and outer layer (approximately 400 A° thick) called the external lamina. These layers contain among other things large amounts of acidic mucopolysaccharides (40) which are therefore anionic at the pH of extracellular space (41). It is reasonable to expect that this area of the cell would be capable of binding significant amounts of calcium and evidence for this has been presented in the literature (42). The calcium stored within the surface layers can be conceived to supply two general systems: (a) a system of pores or conductive channels through the unit membrane and (b) a carrier system capable of moving sodium or potassium outward and calcium inward. The pores are visualized to function as channels for the electrogenic movements of cations (measured by voltage clamp technique) including calcium whereas the carrier system would exchange cations in an electro-neutral manner.

The surface layers (surface coat and external lamina) and their possible role in heart muscle function have been virtually neglected. The major importance of the surface constituents was emphasized by a recent study (2) on sialic acid, which occupies peripheral, terminal position in the oligosaccharide portions of the glycoproteins and glycolipids, that makeup a large component of the cellular surface coat material (43, 44). Langer (2) has observed that removal of sialic acid from the cultured heart cells with highly purified neuraminidase (specific for cleaving sialic acid) markedly increased the cellular calcium exchangeability. It was striking to note that as the membrane became nonselective with respect to calcium permeability following neuramini-

dase treatment, its selectivity for potassium was maintained.

It is now well established that the plasma membrane constitutes the real permeability barrier of the cell and gives the membrane its high electrical resistance and capacitance. Several excellent reviews concerning the membrane molecular organization, ultrastructure, bioelectrical properties and transport function have appeared in the literature (45 - 48). It is also apparent that different membranes may vary substantially in molecular composition, enzymatic activity, transport function, thickness and in the type of image yielded by high resolution electron microscopic techniques, such as negative staining and freeze-etching (45, 49 - 51). Furthermore, the plasma membrane of any given cell type is not necessarily uniform over its whole surface and may be differentiated locally at desmosomes, tight junctions and synapses and may possess a microscopic non-uniformity as in a two-dimensional molecular mosaic.

Each type of membrane contains a characteristic set of complex lipids in a specific molar ratio, which appears to be genetically determined. Plasma membranes, because of their relatively high cholesterol content, are rigidly structured and "lighter" than other types of membranes (52). Furthermore, different molecular species of structural proteins in a given membrane are undoubtedly genetically coded and they may in turn specify the content and ratio of the various lipids of the membrane. It has been suggested (53) that each species of membrane protein may be able to bind selectively a single type of membrane phospholipid.

The most accepted molecular model of the structure of plasma membrane was first proposed by Danelli and Davson (54) and later refined by Robertson (55) as the unit membrane hypothesis. Accordingly, the lipids

of the membrane, which comprise 40 - 50 per cent of its mass, are arranged in a bilayer, with the hydrocarbon chains of the two lipid layers opposed to form a continuous, nonpolar hydrocarbon phase. On either side of the lipid layers are monolayers of protein, which comprise 50 - 60 per cent of the membrane mass. The unit membrane hypothesis was originally postulated to account for the basic structure of all types of membrane; however, membranes may change locally in structure as a function of their activity. There is one feature of the unit membrane structure, namely the lipid bilayer, which still best accounts for the characteristic permeability and electrical properties of plasma membranes. Nuclear magnetic resonance measurements have revealed that the proteins are relatively fixed in the membrane structure but that the lipids have considerable freedom of movement (56, 57). One of the strongest pieces of evidence in support of the lipid bilayer model is the fact that, in the complete absence of protein, phospholipids in aqueous systems form bilayers spontaneously in the form of flat micelles as large closed vesicles. Most pertinent are the studies by Mueller et al. (58) and by Maddy and coworkers (59) who have shown that phospholipid bilayers may be formed in apertures separating two aqueous phases. These bilayers have low permeability to polar solutes, high permeability to water and extremely high electrical resistance and capacitance comparable with that of the plasma membrane. Such lipid bilayers can also be made electrically excitable in the presence of an ion gradient (60).

The studies of Fleischer et al. (61, 62) have emphasized the important role of protein in the basic membrane structure by showing that the electron microscopic appearance of the mitochondrial membrane remained relatively unchanged after extraction of most of the mitochondrial lipid.

A direct approach to the determination of conformation of membrane proteins by physical studies on intact cell membranes has been attempted by Wallach and Zahler (63) with plasma membranes of Ehrlich ascites carcinoma cells, by Maddy and Malcolm (64,65) and Lenard and Singer (66) with membrane preparations from human red cells, by Ke (67) with chloroplast lamellae, and by Urry et al. (68) with mitochondria and submitochondrial particles. Infra-red spectroscopic examination indicated that little or none of the protein existed in the β-conformation (64-66) whereas optical rotatory dispersion and circular dichorism studies (63, 66, 68) revealed that an appreciable percentage of the membrane protein may be present as an α-helix. On the basis of the rotatory dispersion and circular dichroism studies it was suggested (63,66) that the α-helix position of membrane proteins is located in a special local environment within the hydrophobic position of the basic lipid bilayer, implying a large degree of hydrophobic bonding between proteins and lipids (63) or between nonpolar positions of the adjacent helical regions of protein (66).

Although the concept of a subunit structure for biological membranes had been suggested by several electron microscopists, this model has been articulated by Green and Perdue (69), who proposed that biological membranes are repeating arrays of individual lipoprotein subunits rather than continuous bimolecular leaflets of phospholipids with proteins present mainly on the surfaces of the bilayers. The yield that functional proteins are not distributed at random along the membrane structure is consistent with current ideas of organized enzyme systems which require the co-operative action of several proteins. It is unlikely that proteins involved in several membrane functions fall in this category, including enzymes of the electron transport chain, proteins involved in membrane

transport and enzymes involved in biosynthesis of macromolecular constituents of the membrane.

C. Sarcolemmal Ca^{2+}/Mg^{2+} ATPase:

Plasma membrane is considered to contain various enzyme systems which may be involved in the regulation of ionic permeability and modulation of myocardial contractility. Cytochemical evidence suggests the localization of both $Na^+ - K^+$ ATPase and adenylate cyclase in heart cell membrane (70, 71). The cardiac membrane fractions enriched in sarcolemma obtained by employing different procedures (72 - 78) also showed high specific activities of $Na^+ - K^+$ ATPase and adenylate cyclase. It has been suggested by various investigators that both adenylate cyclase and $Na^+ + K^+$ ATPase are intimately involved in the regulation of heart function (79). On the other hand, most of the research in the field of plasma membrane has been concerned with the number and kind of enzyme ATPases, their distribution, their functions particularly in relation to cation transport, nature of the cation activation, substrate specificity, mechanisms of enzyme reaction, purification and isolation. In mammalian tissues, at least three different enzyme activities are recognized on the basis of cation activation and inhibition, sensitivity to cardiac glycosides and substrate specificity. These are Mg^{2+} stimulated ATPase, the $Na^+ - K^+$ ATPase, which is sensitive to cardiac glycosides, and the Ca^{2+} stimulated ATPase. The widespread distribution of both the Mg^{2+} ATPase and $Na^+ - K^+$ ATPase has been demonstrated in a variety of tissues from different sources (80 - 85). On the other hand, very little information concerning the plasma membrane Ca^{2+} stimulated ATPase is available in the literature.

Recently, various investigators have reported the isolation of

heart sarcolemma by different methods (72, 73, 75 - 78, 86, 87). These membrane preparations have been shown to exhibit high specific activities of different enzymes including Ca^{2+}/Mg^{2+} ATPases. It should be pointed out that all these studies were mainly concerned with establishing the purity of the sarcolemmal preparation by ruling out the contamination due to mitochondria, microsomes and myofibrils. Furthermore, while a considerable amount of information concerning the properties of the heart sarcolemmal adenylate cyclase and Na^{+} - K^{+} ATPase has accumulated in the literature, relatively little is known about the sarcolemmal bound Ca^{2+} ATPase and Mg^{2+} ATPase. It should be noted here that the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase being referred here is distinctly different from the heart sarcolemmal Ca^{2+} - stimulated Mg^{2+} dependent ATPase, which serves as a "calcium pump" for calcium efflux (73, 74, 88, 89). The sarcolemmal Ca^{2+} - stimulated Mg^{2+} dependent ATPase, like the microsomal Ca^{2+} stimulated Mg^{2+} dependent ATPase, utilizes Mg ATP as a substrate and requires micromolar concentrations of calcium for activation. On the other hand, the sarcolemmal Ca^{2+}/Mg^{2+} ATPase is considered to represent an ATP hydrolyzing system which is activated by most divalent cations.

The presence of Ca^{2+}/Mg^{2+} ATPase in sarcolemma is not limited to the myocardium because this enzyme system has also been shown to be present in cell membrane preparations from skeletal muscle (90, 91), smooth muscles (92 - 95), liver (96, 97), kidney (98), pancreas (99), submandibular glands (100), mammary gland (101), neuroblastoma cells (102), mast cells (103), bone cells (104), and sciatic nerve (105). Although the exact role of the mammalian cell membrane Ca^{2+}/Mg^{2+} ATPase in different cellular functions is not clear, its involvement in the so-

called passive influx of calcium has been suggested by some investigators. On the other hand, Ca^{2+}/Mg^{2+} ATPase of the bacterial and chloroplast cell membranes has been shown to possess similarities with mitochondrial ATPase (106, 107). These ATPases are considered to be involved in functions such as oxidative phosphorylation, photophosphorylation, active proton translocation, exocytosis and receptors for divalent cations in bacterial sensing (108 - 111). A great deal of work on the bacterial cell membrane Ca^{2+}/Mg^{2+} ATPase has appeared in the literature and this enzyme system infact has been isolated and purified by several procedures (106, 107). Solubilized bacterial divalent cation ATPase has been shown to cause a marked increase in the electrical conductance of bimolecular lipid membrane (112) and has been considered to serve as an ionophore (113). No such information with respect to the sarcolemmal Ca^{2+}/Mg^{2+} ATPase is still available in the literature. Furthermore, it is not clear whether the sarcolemmal ATP hydrolyzing ability in the presence of Ca^{2+} or Mg^{2+} is due to the same enzyme complex. It is also not known whether the sarcolemmal Ca^{2+}/Mg^{2+} ATPase is similar to such enzyme systems present in other organelles, particularly in mitochondria and sarcoplasmic reticulum.

It is well known that there is present in the heart cell a Ca^{2+} - ATPase located in the plasma membrane (76, 114, 115). Evidence concerning the presence of extracellular divalent cation ATPase activity in the intact cardiac cells has been presented by other investigators (116, 117). The full activation of this enzyme system is seen at millimolar concentrations of calcium. This would tend to rule out the possibility that the function of Ca^{2+} ATPase in the heart cell is that of a cation pump, which ordinarily extrudes Ca^{2+} ions from the cell by expenditure of ATP. It is

possible that the true orientation of the Ca^{2+} ATPase for divalent cationic site is towards the exterior of the cell, but that the enzyme molecules are not fixed rigidly in this configuration, so that presentation of divalent cation to the external surface causes a reversal of their positions. This enzyme system may thus be able to utilize ATP which is present either in the cytoplasm or is compartmentalized in the cell membrane. Alternatively, the catalytic site for this enzyme system may be directed towards the exterior of the heart cell and under this situation ATP for the enzymatic reaction must be made available outside the cell. In this regard, it should be noted that the results on the effects of ATP on cation permeability in erythrocytes, mast cells and chick embryo fibroblasts have revealed the true site for ATP to be located towards the exterior of the cell (118, 119). The availability of ATP to such ecto-enzyme systems can be seen as a major problem.

The involvement of an ATPase enzyme system in any process must mean that energy is indeed expended. In this regard it should be noted that a contractile protein with the characteristics of smooth muscle actomyosin Ca^{2+} - activated ATPase has been found to be present in different types of non-muscle cells (103, 120, 121). The heart cell sarcolemma may also possess a similar contractile protein system which exhibits Ca^{2+}/Mg^{2+} ATPase activity. Although it seems unlikely that the cell is going to encounter extracellular concentrations of ATP in the millimolar range in vivo , various mammalian cells have been shown to be capable of synthesizing ATP on their outer surface (122). In fact the local concentration of ATP in the cell membrane is believed to be about 2 mM (123). Furthermore glyceraldehyde 3 - phosphate dehydrogenase, phosphoglycerate kinase, adenylate kinase and creatine phosphokinase have

also been shown to be ectoenzymes capable of synthesizing ATP extracellularly (124 - 127). It is possible therefore that such enzymes could provide the necessary substrate for the ATPase identified in the heart sarcolemma. It is emphasized here that it is not our intention to undermine the availability of intracellular ATP to ATPase located in heart sarcolemma. At any rate, the exact nature and orientation of the Ca^{2+}/Mg^{2+} ATPase as well as the exact source of ATP for this system remains to be a matter of speculation at this time.

Recently, opening of the slow channels, through which the inward Ca^{2+} current during the action potential plateau traverses the heart sarcolemma, has been shown to require metabolic energy in the form of ATP (128). If this is the case then an ATPase system similar to the sarcolemmal Ca^{2+}/Mg^{2+} ATPase can be conceived to be involved in the opening of calcium channels in the myocardium. This view is supported by the fact that Co^{2+}, Mn^{2+} and Ni^{2+}, which have been shown to depress calcium currents, have also been found to decrease the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase activity (129). Likewise, different antiarrhythmic agents and β-adrenergic blocking agents, which depress myocardial contractility, have been demonstrated to decrease the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase activity (130, 131). The sarcolemmal Ca^{2+}/Mg^{2+} ATPase activity has also been observed to be less in the failing hearts in comparison to the normal myocardium (132 - 135). It appears that the Ca^{2+}/Mg^{2+} ATPase may represent a biochemical correlate of calcium channels in heart sarcolemma or may be intimately involved in opening these channels or gates for the entry of calcium in the myocardium (136). Although depolarization of myocardium is generally considered to increase the permeability of the cell to calcium by opening calcium channels in the

sarcolemma, the exact mechanism involved in opening of the calcium channels is not understood. Thus any information regarding this mechanism would extend our knowledge and the proposed implication of the Ca^{2+}/Mg^{2+} ATPase in this event appears to have an attractive lead for a detailed investigation. The involvement of the sarcolemmal Ca^{2+} ATPase in opening calcium channels and subsequent entry of calcium into the myocardium upon depolarization or even upon hormonal activation should not be viewed as a phenomenon analogous to active calcium transport across different membrane systems. On the contrary, the movement of calcium from the extracellular fluid into the cell is a passive process in the conventional sense and it is the opening of the calcium channels, which requires energy through the participation of the sarcolemmal Ca^{2+} ATPase system.

D. Role of Phospholipids in Membrane Bound Enzyme Activities:

In view of the fact that membrane bound enzymes are embedded in membrane phospholipids, alterations in the composition of these phospholipids can be conceived to produce changes in the enzyme activity. Fleishers and Klouwen (137) demonstrated an essential role for phospholipids in several of the reactions of the mitochondrial electron transport system and likewise Green and his associates (138 - 140) showed a phospholipid requirement for the activity of β-hydroxybutyrate dehydrogenase of beef heart mitochondria. A role of phospholipid in the activity of cytochrome oxidase was also suggested by some investigators (141 - 143) and a stimulation of the activity of purified mitochondrial succinate dehydrogenes by phospholipids was reported (144). It is assumed that lipid interacts directly with the enzyme protein in these reactions and that the lipid protein complex is the enzymatically active species. In the

case of integrated enzyme complexes such as the electron transport chain, it is possible that lipid or structural protein or both play a dual role, activating the individual enzymes and also directing the sequential adjacent localization of membranes of the enzyme chain within the membrane structure.

Bacterial systems have also provided several examples of lipid-enzyme interactions. In one such example a requirement for phosphatidyl ethanolamine has been shown in two of the enzyme reactions involved in biosynthesis of the cell envelope lipopolysaccharide of Salmonella typhimurium (145). In this case an interaction was repaired between phospholipid and lipolysaccharide to prevent the binding of enzyme to its substrate. In mycobacterium avium, Tabari (146) has reported a phospholipid requirement for activity of an FAD-dependent malate dehydrogenase. Recently it has been shown that the ATPase from E-coli is dependent on the membrane, although loosely bound to it (147). Removal of phospholipids from the ATPase preparation leads to a loss of hydrolytic activity. For full activity at least 100 molecules of phospholipids, mainly phosphatidyl ethanolamine per molecule of ATPase are needed; some of these phospholipid molecules are strongly bound to the ATPase. Kobayashi and Auraku (148) reported that highly purified E-coli ATPase contained 1 - 2 mol of phospholipid per mol of the ATPase. Solubilization of membrane - bound E-coli ATPase also caused a loss of activity (149). The lost activity was restored by the addition of original membrane fragments (149). Addition of soybean phospholipids to an E-coli ATPase preparation caused extensive stimulation of ATPase activity (150). It has been further reported that cardiolipid is essential for the activity of the E-coli ATPase, which is very similar to the

mitochondrial ATPase, since destruction of cardiolipid is accompanied by a reduction of the ATPase activity.

The essential role of phospholipids in the activation of particulate mitochondrial ATPase and in the restoration of oligomycin sensitivity is well documented (151 - 154). Activation of the enzyme complex is produced either by a crude preparation of phospholipids of different origin or by several individual phospholipids. Although several reports (155 - 157) indicate the requirement of phospholipids by the $Na^+ - K^+$ ATPase enzyme complex, the nature of the phospholipids involved is not known yet. Some evidence (155 - 157) indicates the essential role of phosphatidyl serine in the activation of $Na^+ - K^+$ ATPase. However, the addition of phosphatidyl serine to phospholipid-depleted preparations made by extraction with deoxycholate (158) resulted in low specific activity, giving the impression that in the full active enzyme, some other phospholipid might be involved (159). Recently one of these preparations was further purified but was stimulated only two fold by phosphatidyl serine. In view of the cation binding property of negatively charged phospholipids and the apparent specific activating effect of phosphatidyl serine on transport ATPase, it has been proposed (160) that this phospholipid takes active part directly in the movement of cations across the cell membrane. Cardiolipid on the other hand is known to be effective in preserving the activity of succinate dehydrogenase (161), activated the mitochondrial ATPase but elicited low oligomycin sensitivity and was completely inactive for the reconstitution of ubiquone reductase activity. Bovine phosphatidyl ethanolamine was fully active in the stimulation of reconstituted succinate ubiquinone-reductase and of oligomycin-sensitive ATPase. Soybean lecithin also

stimulated the mitochondrial ATPase and restored high oligomyocin sensitivity.

The involvement of phospholipids on the lipoprotein-nature of the erythrocyte membrane bound ATPase enzyme system has been reported by a number of investigators during the last few years. At first this involvement has been demonstrated by a reduction of the ATPase activity after treatment of the enzyme preparation with phospholipase A (162) or phospholipase C (163). Several workers have attempted to restore the ATPase activity by adding back phospholipids (164 - 167, 155 - 158). Although, there was no or little agreement in the literature on the nature of the lipid bringing about a reactivation, the phosphatidyl serine was found out to be the one which is more active in restoring the activity of $Na^+ - K^+$ - ATPase. However, Hegyvory and Post (167) demonstrated reactivation of $Na^+ + K^+$ - ATPase from kidney membrane, after phospholipase A treatment, by the addition of a commercial soybean extract, asolectin. It was further emphasized that the ATPase activity could be restored as long as the enzyme has been partially inactivated. Israel (168) demonstrated that the $Na^+ - K^+$ ATPase from untreated beef brain microsomes could be activated by the addition of phosphatidyl serine to the ATPase incubation medium. Wheeler and Whittam (159) also reported that the $Na^+ + K^+$ ATPase activity present in the membrane containing fraction of ox-brain was raised by phosphatidyl serine in the ordinary enzyme preparation before it had been treated with other agents. This effect is explained by the authors that some phospholipid may be lost during the preparation of the enzyme. This suggestion was however not confirmed by Roelofsen et al. (169), who suggested that removal of the "loosely bound" phospholipids does not alter ATPase activities present in the erythrocyte

membrane. Tanaka and coworkers (157, 160, 170), demonstrated that the enzyme prepared from cerebral cortex of beef cattle by treatment of deoxycholate could be reactivated by addition of acidic phospholipids such as phosphatidic acid, phosphatidyl inositol and phosphatidyl serine, whereas neutral lipids such as lecthin and phosphatidyl ethanolamine were found to be ineffective in this respect. It was suggested by these authors that the essential structures needed for activation are a phosphate group plus one or two fatty acyl residues.

The lipid dependences of the Ca^{2+} + Mg^{2+} ATPase in the human erythrocyte membrane has also been demonstrated recently (171 - 173). Coleman and Bramley (171) have shown a loss of (Ca^{2+} +Mg^{2+}) - ATPase activity upon treatment of ghosts with a partially purified preparation of phospholipase C from clostridium Welchii as well as a subsequent reactivation of the enzyme by the addition of phospholipids. However these investigators did not study the role of various phospholipids in restoring the enzyme activity. Roelofsen and Schatzmann (174) have shown the complete preservation of the (Ca^{2+} - Mg^{2+}) - ATPase activity after treatment of intact erythocytes with Naja naja phospholipase A_2 and sphingomyelinase C, suggesting that the ATPase does not require the presence of the original phospholipids of the outer leaflet and hence the intact phospholipid bilayer. On the other hand, they found a dramatic decrease in (Ca^{2+} + Mg^{2+}) - ATPase activity, when the glycophospholipids were almost quantitatively converted into their lyso-derivaties and free fatty acids. It has been claimed recently by Quist and Raufogalis (173) that phospholipase A_2 treatment affects only the so-called low affinity (Ca^{2+} + Mg^{2+}) - ATPase in erythocyte ghosts and not the high affinity one. Exhaustive hydrolysis of glycerophospholipids with B cereus

phospholipase, however, abolished the (Ca^{2+} - Mg^{2+}) - ATPase activity completely (174).

In addition to providing Ca^{2+} binding sites for the accumulated Ca^{2+}, phospholipids are required for the ATPase activity and Ca^{2+} transport in the sarcoplasmic reticular (microsomal) membrane (175 - 179). Phospholipase C (Clostridium Welchii) hydrolyzed about 80 - 90% of the lecithin of microsomal membranes to phosphoryl choline and diglycerides (175, 176, 179-181) resulting in nearly complete inhibition of ATPase activity (180), relaxing activity (181), Ca^{2+} transport (175, 176, 179) and ATP-ADP exchange activity (182). The depressed ATPase activity and Ca^{2+} transport of phospholipase C treated microsomes can be reactivated by ultrasonic dispersion of lysophosphatidyl choline and synthetic and natural phosphatidyl choline preparations, whereas phosphatidyl serine and phosphatidyl ethanolamine were less effective (175, 176, 179). Prolonged digestion (10 - 15 hr) with phospholipase C resulted in preparations which are not readily reactivated by phospholipids. Similar reactivation of ATPase activity and Ca^{2+} transport is obtained with micellar suspension of synthetic dipalmitolylecithin containing only saturated fatty acid and lecithin obtained from egg, calf brain or skeletal muscle microsomes, which contain a sizable portion of unsaturated fatty acids. These observations suggest that the nature of the fatty acids mainly of phospholipids may not be a dominant aspect of their involvement in the Ca^{2+} transport and ATPase activity of skeletal muscle microsomes. This conclusion was further supported by the experiments of Yu et al. (183) demonstrating that the rate and extent of Ca^{2+} uptake of skeletal muscle microsomes isolated from essential - fatty acid deficient rats were not different from the control values.

Phospholipase A treatment of microsomes in the presence of serum albumin did not inhibit ATPase activity, although up to 50% of the phospholipids were hydrolyzed (183). The ineffectiveness of phospholipase A treatment in inhibiting ATPase activity was also shown by Martonosi (175, 176, 179). Phospholipase D, on the other hand, hydrolyzed a major portion of the microsomal lecithin into phosphatidic acid and choline without inhibition of the ATPase activity and Ca^{2+} transport (170, 183). In this regard it should be pointed out that phosphatidic acid, was found to partially restore the Ca^{2+} transport and ATPase activity of phospholipase C - treated microsomes (180).

From the above mentioned literature, it is clear that a great deal of work has been carried out concerning the role of phospholipids in Ca^{2+} transport and ATPase activities of various membrane systems, relatively little information is available with respect to the sarcolemmal preparations. Sulakhe et al. (184) have shown that phospholipase A anc C inhibited Na^{+} - K^{+} ATPase activity in heart sarcolemma. Phospholipase C has also been reported to inhibit calcium binding by liver membrane, skeletal muscle sarcolemma and heart sarcolemma (185 - 187), whereas, not much effect was observed with phospholipase A and D (187). Practically no work has been reported concerning the role of phospholipids in Ca^{2+}/Mg^{2+} ATPase activities of the heart sarcolemma.

E. Role of Membrane Protein Integrity in Ca^{2+} Transport and ATPase Activity:

Proteins which exist in the membrane are considered to play an important role in the transport process (188 - 191) and in bacterial membranes these are concerned with energy transducation in the transport process (192). Sarcoplasmic reticulum membranes have been studied extensively in order to find out the protein structure - function

relationships since these display only one major activity, i.e. Ca^{2+} uptake coupled to ATP hydrolysis (193 - 196). It has been reported by various investigators that the Ca^{2+} transport activity of sarcoplasmic reticulum membranes is inhibited by brief digestion with trypsin and other proteolytic agents (197 - 200). This was accompanied with a transient increase in the Mg^{2+} + Ca^{2+} - activated ATPase activity, a decrease in the turbidity of microsome suspension and release of 10 - 30% of the microsomal proteins in a form which was not sedimentable on centrifugation at 100,000 g. The ATPase activity remained associated with the membrane fraction even after trypsin digestion causing the disappearance of the 40°A subunits from the surface of the microsomes (197 - 199). These data have been interpreted to suggest that the ATPase enzyme connected with the Ca^{2+} transport is embedded in the phospholipid core of the microsomal membrane and is either insensitive or inaccessible to trypsin and other proteolytic enzymes.

Prolonged digestion of the microsomal membrane with trypsin resulted in decline of ATPase activity to about one-fourth of the initial activity together with marked changes in membrane morphology like the removal of surface particles; conditions which stabilized the ATPase activity were shown to protect the surface particles (201). From this study it was concluded that surface particles are a functional part of the microsomal ATPase protein (201). This view was further supported by earlier observations of Hasselbach and Elfin (202) who showed that azoferritin, specifically bonded to sulfhydryl groups at the active site of the ATPase was bound to structures on the membrane surface. This ATPase was further characterized by using gel electrophoresis, electron microscopy, negative staining and freez-etching. It was shown further that the ATPase of sarcoplasmic reticulum may be identified with 106,000 MW protein component

(203, 204) which is partially exposed to trypsin digestion on the outer surface of the vesicles. A portion of the enzyme is embedded within the membrane and is more resistant to trypsin digestion. The outer segments of 106,000 MW protein appeared as granules on negatively stained vesicles and the inner segments as particles of the concave freeze fracture faces. The tryptic fragments of the ATPase molecule remained joined to each other and to the membrane through multiple weak interactions retaining native conformation and enzyme activity. However, prolonged trypsin digestion caused enzyme inactivation. It has thus been proposed (204) that the outer membrane leaflet of the sarcoplasmic reticulum membrane is composed mostly of ATPase, while the inner leaflet is prevalently a lipid monolayer.

It should be noted that trypsin has been shown to stimulate Ca^{2+} ATPase and Mg^{2+} ATPase activities of E. Coli and other bacteria (205). On the other hand, a decrease in Na^{+} - K^{+} - ATPase activity and calcium binding in heart sarcolemma by trypsin treatment has also been reported by some investigators (184, 187). Thus modification of the membrane protein composition by treatments such trypsin digestion can be seen to alter the membrane bound enzyme activities depending upon the experimental conditions as well as the nature and location of the enzyme system in the membrane. It is also pointed out that no report concerning the effects of trypsin on the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase has yet appeared in the literature.

III. MATERIALS AND METHODS

Healthy albino rats weighing about 300 g were used in this study. All the chemicals used in this study were of analytical grade. Phospholipase A (from Naja-naja venom), phospholipase C (from clostridium welchii), trypsin inhibitor (from Soybean), cyclic 3',5' adenosine monophosphate, adenosine triphosphate and protein kinase (from beef heart) were obtained from Sigma. Trypsin (pancreas), phospholipase D(cabbage) were purchased from Calbiochem. Synthetic DL - α - dipalmitoyl lecithin, L - α - palmitoyl lysolecithin and DL - α - dipalmitoyl phosphatidic acid, capric acid, lauric acid and linoleic acid were purchased from Sigma. Stearic acid, palmitic acid and oleic acids were obtained from Applied Science Laboratories.

A. Isolation of Heart Membranes:

1. Isolation of heart sarcolemmal fraction

Rats were decapitated and their hearts were quickly removed and placed in ice-cold 10 mM Tris-HCl buffer, pH 7.4. The ventricles were washed thoroughly, diced with a pair of scissors, homogenized with 10 vol. of 10 mM Tris-HCl pH 7.4 containing 1 mM ethylene-diaminetetra-acetate disodium (EDTA) in a Waring blendor for 1 min (30 sec x 2 with an interval of 30 sec). The homogenate was filtered through a gauze and centrifuged at 1,000 x g for 10 min. The sediment was suspended in 20 - 25 vol. of 10 mM Tris-HCl buffer, pH 7.4 and stirred in the cold room for 30 min and centrifuged at 1,000 x g for 10 min. The residue was then suspended in 20 - 25 vol. of Tris buffer, pH 8.0, stirred for 30 min and centrifuged at 1,000 x g for 10 min. The sediment was suspended in 10 mM Tris-HCl buffer, pH 7.4, stirred for 30 min and centrifuged at 1,000 x g for 10 min; this step was then repeated again. The sediment was suspended in 20 - 25 vol. of 10 mM Tris-HCl, pH 7.4, extracted with 0.4 M LiBr for 45 min and

centrifuged at 1,000 x g for 10 min. This sediment was then suspended in Tris-HCl, pH 7.4, stirred up for 10 min and centrifuged at 1,000 x g for 10 min. The sediment was further extracted with 0.6 M KCl containing 10 mM Tris-HCl buffer, pH 8.0, washed with 10 mM Tris-HCl, pH 7.4, suspended in 1 mM Tris-HCl, pH 7.0, and employed for biochemical studies. All the above steps were performed at 0 - 4°C. This procedure is essentially similar to that described by McNamara et al. (78).

2. Isolation of mitochondrial fraction

Hearts were thoroughly washed in 0.25 M sucrose containing 1 mM EDTA, pH 7.0. The tissue was homogenized in 10 vol. of 0.18 M KCl, 10 mM EDTA, 0.5% albumin (fatty acid free), pH 7.4, in a Teflon homogenizer till complete cellular disruption was obtained. The homogenate after filtering through two layers of gauze was centrifuged at 1,000 x g for 20 min to remove cell debris, nuclei and myofibrils. The supernatant was filtered through two layers of gauze and was centrifuged at 10,000 x g for 20 min. The resulting mitochondrial pellet was resuspended in homogenizing medium and centrifuged at 10,000 x g for 10 min. The washing procedure was repeated twice to remove cellular contaminants adhering to the mitochondria. The final mitochondrial pellet was suspended in 1 mM Tris-HCl, pH 7.0. This method for isolating the heart mitochondria is similar to that described by Sordahl and Schwartz (206).

3. Isolation of heart microsomal fraction

Hearts were thoroughly washed with 0.25 M sucrose containing 1 mM EDTA, pH 7.0. The tissue was homogenized in 10 vol. of medium containing 10 mM sodium bicarbonate, 5 mM sodium azide and 15 mM Tris-HCl, pH 6.8, in a Waring blendor for 45 sec. The homogenate was filtered through four layers of gauze and centrifuged at 10,000 x g for 20 min to remove

cell debris, nuclei, myofibrils, and mitochondria. The residue was discarded and the supernatant was spun at 40,000 g for 45 min. The sediment thus obtained was washed thoroughly, suspended in 0.6 M KCl, containing 20 mM Tris-HCl, pH 6.8 and centrifuged at 40,000 x g for 45 min. This procedure was repeated twice and the final pellet was suspended in 1 mM Tris-HCl, pH 7.0. This method of isolation for the heavy microsomes is essentially similar to that described by Harigaya and Schwartz (207).

B. SDS Gel Electrophoresis:

Sodium dodecyl sulfate (SDS) polyacrylamide gels were run in the buffer system of Weber and Osborn (208).

1. Preparation of protein solution

The membrane proteins were incubated at 37°C for 1 hr in 0.01 M sodium phosphate buffer, pH 7.0, 1% SDS and 1% β-mercaptoethanol. The protein concentration was normally between 0.2 and 0.6 mg per ml.

2. Preparation of gels

Gel buffer contained 7.8 g $NaH_2PO_4H_20$, 38.0 g of $Na_2HPO_47H_2$) and 2 g of SDS per litre. Seven % acrylamide and 0.42% methylenebis acrylamide were used for preparing tube gels. The glass gel tubes were 10 cm long with an inner diameter of 6 mm. For a typical run of 12 gels, 15 ml of gel buffer were deaerated and mixed with 13.5 ml of acrylamide solution. After further deaeration, 1.5 ml of freshly made ammonium per sulphate (15 mg per ml) and 0.045 ml of N, N, N^1, N^1 - tetramethylecylenediamine were added. Before the gel hardened a few drops of water were layered on top of the gel solution. After 10 to 20 min when the gels had solidified, the water was removed.

3. Preparation of samples

For each gel, 25 µl of mercaptoethanol, 25 µl tracking dye and 50 µl of protein

sample (preincubated) were mixed in a small test tube. After mixing, the sample (25 - 50 µl) was applied on the gels which were then covered carefully with 1 to 2 drops of glycerol. Gel buffer diluted 1 : 1 with water was layered on top of each sample to fill the tubes. The two compartments of the electrophoresis apparatus were filled with gel buffer diluted 1 : 1 with water. Electrophoresis was performed at a constant current of 2.5 mA per gel with the positive electrode in the lower chamber. When the tracking dye reached at the end of the gel, the electrophoresis was stopped. The gels were removed from the tubes by squirting water from a syringe between gel and glass wall.

4. Staining and destaining

The gels were stained with coomassie brillant blue in methanol and glacial acetic acid. Staining was done for 2 hr at room temperature, the gels were washed with distilled water and then destained in 7% acetic acid solution in a destainer. The gels were stored in 7% acetic acid solution and scanned with a Gilford linear transport system using 0.80 absorbance units for full scale deflection.

C. Measurement of Phospholipid Composition:

1. Extraction of lipids

Lipids were extracted by the method of Folch et al. (209). The membrane fractions were homogenized with 2 : 1 chloroform-methanol mixture (v/v) to a final dilution of 20 - fold the volume of the tissue sample in a glass homogenizer. The homogenates were then allowed to stand for 1 hour with occassional mixing and then filtered through a fat free paper into a glass stoppered tube. The homogenizer tube was rinsed with the same solvent and the filtrate was collected. Lipid extract was washed with acidified distilled water (0.5% H_2SO_4) in an amount equal to 20% of the

volume of the extract. It was shaken vigorously, allowed to settle and the upper phase was suctioned off. The interface was washed twice with 1 ml aliquot from the upper phase of a solution of 8 parts chloroform: 4 parts methanol : 3 parts water and then suctioned off 1/2 of the upper phase. The tubes containing the lipid extract were placed in a water bath at 45 - 50°C and samples were evaporated to dryness in the atmosphere of 100% nitrogen. The residue was then dissolved in chloroform: methanol mixture for further analysis.

2. Fractionation on silicic acid (Unisil)

Lipids can be separated into three major classes namely - less polar lipids (cholesterol, cholesterol esters, di and triglycerides), glycolipids (sulfatides plus cerebrosides) and phospholipids by silicic acid column fractionation using the method outlined by Sun and Horrocks (209a). The method consisted in dissolving the lipids into 10 ml of chloroform and applying them to a 1 x 4 cm column containing 5 grams of Unisil. The Unisil column was prepared by suspending the silicic acid in chloroform and packing it into a glass tube (1 cm in diameter). The column was washed with 100 ml of methanol and then 100 ml of chloroform. Subsequently, the lipids were dissolved in a small volume of chloroform and applied to the column. Less polar lipids were eluted with 50 ml of chloroform, glycclipids were eluted with 50 ml of chloroform : acetone (1 : 1,v/v) and then 50 ml of acetone, and phospholipids were eluted with 100 ml of methanol. The phospholipids thus eluted were evaporated to dryness in the atmosphere of 100% nitrogen. The residue was dissolved in the chloroform: methanol mixture.

3. Thin layer chromatography

Quantitative analysis of phospholipids by thin layer chromatography was performed by the method of Parker and Peterson (210). The

silica gel plates were activated for 1 hr at 110°C just before use. Phospholipids were applied to the silica gel plate with a microliter pipet. Duplicate aliquots of the same amount of phospholipids were placed in test tubes for determination of the total phosphorus. The plates were subjected to ascending chromatography by placing them in a tank which contained 184 ml of chloroform - methanol - acetic acid-water, 25: 15: 4: 2. The solvent was allowed to rise within 0.5 cm of the top of the adsorbent. Average running time was 1 - 1.5 hr. For the analysis of phospholipid phosphorus, the plate was exposed to iodine vapor and the spots were immediately outlined with the point of a needle. The phospholipids were identified by simultaneous chromatography of reference phospholipids.

4. Analysis of phospholipid-phosphorus

After the iodine had evaporated from the plate, each outlined spot was scraped, collected on a sheet of glazed paper and transferred to a pyrex text tube. Adjacent areas of blank silica gel corresponding in size and position to the areas containing phospholipid were also scraped into test tubes. Concentrated sulphuric acid, 0.5 ml was added to all test tubes, including tubes to contain reagent blank and inorganic phosphorus standards. One - 2 crystals of carborundum were also added to each tube. The tubes were placed in a heating block and digested for 3 hr at 250°C. After 2 hr of digestion, the tubes were gently swirled to break up clumps of silica gel. Following digestion the tubes were removed from the heating block, allowed to cool, and 2 - 3 drops of 30% hydrogen peroxide were added. The tubes were then returned to the heating block and digested for one hr at 160°C.

Phospholipid phosphorus was determined by the method of Fiske and Subbarow (211). To all the tubes containing silica gel, the reagent blanks

and "total phospholipid" aliquot, 5 ml of distilled water was added followed by 1.0 ml of 2.5% ammomium molybdate. The contents were mixed and 0.5 ml of 1 - amino - 2 - napthol - 4 - sulphonic acid (ANSA) reagent was added. The tubes were stoppered, contents mixed and placed in a boiling water bath for 7 min. After centrifugation at 300 x g for 40 min, the samples were transferred to the cuvettes with the aid of capillary pipettes and read at 650 mμ.

D. Electron Microscopic Examination:

The membrane fractions isolated by the procedures described above were examined electron microscopically. The pellets were fixed in 1% glutaraldehyde in a 0.1 M phosphate buffer pH 7.4 for 16 hr. These specimens were further fixed with 1% osmium tetraoxide, dehydrated in a graded ethanol series and embedded in Epon 812 according to the method of Luft (212). Sections of these specimens were cut with a Porter - Blum MT-II ultra-microtome using glass knives, stained with uranly acetate and lead citrate, and examined with a Zeiss electron microscope (EM 9S).

E. Negative Staining:

Negative staining was accomplished by diluting the samples with 2% phosphotungstic acid adjusted to pH 6.8. A drop of the diluted sample was placed in a grid covered with a thin film of formvar reinforced with evaporated carbon. After 20 sec, the drop was blotted off with filter paper and the grid was allowed to air dry. The specimens were examined in a Zeiss electron microscope (EM 9S). This method is essentially similar to that described by Parson (213).

F. Trypsin Digestion:

In some experiments sarcolemmal membrane (2 mg/ml) was digested with various concentrations of trypsin at room temperature for different time intervals in a medium containing 50 mM Tris-HCl, pH 7.5, and 20 mM

KCl. The reaction was stopped by the addition of 2 - 3 fold of soybean trypsin inhibitor. The tubes were centrifuged at 1,000 x g for 10 min. The supernatant was saved for the determination of ATPase activity. The residue was washed twice with 1 mM Tris-HCl, pH 7.0, and finally suspended in the same buffer and aliquots were used for ATPase activity, protein composition as determined by sodium dodecyl sulfate gel electrophoresis and morphological studies. As indicated in the text, the trypsin digestion, in some experiments was done in a medium containing various ligands such as $CaCl_2$, $MgCl_2$ or ATP in addition to Tris-HCl and KCl.

G. Phospholipases A,C and D Treatment:

All phospholipase incubations were carried out at room temperature. Heart sarcolemmal fraction was treated with different phospholipases A,C, D for different time intervals at a room temperature in a medium containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl and 1 mM $CaCl_2$. The concentration of phospholipase A varied from 10 µg to 250 µg/mg of membrane protein whereas the concentrations of phospholipase C and D were from 100 µg to 1 mg/mg of membrane protein. At the end of the treatment, the reaction was terminated by the addition of 2 mM EGTA. The contents were centrifuged, the pellet was washed twice with 1 mM Tris-HCl buffer, pH 7.0. The final suspension was made in the above buffer. Aliquot of the suspension were used for the determination of ATPase activities and morphological examination. For reconstitution studies, different concentrations of various phospholipids such as phosphatidic acid, lysolecithin and lecithin were added to the assay systems after sonic disruption with a Branson ultrasonic apparatus for 1 to 5 min in ice at 2.5 amps (179).

H. Phosphorylation of Heart Sarcolemma:

Heart sarcolemma was incubated in the presence of 5 mM Mg ATP,

20 µg protein kinase mg membrane protein 10^{-6} M cyclic AMP at 25°C for 5 min, centrifuged and gently washed with 1 mM Tris-HCl buffer pH 7.0. The residue was suspended and employed for the determination of ATPase activities. This method of phosphorylating sarcolemma is similar to that of Sulakhe et al. (186).

I. Biochemical Studies:

All the cellular fractions employed in this study were used within 1 - 2 hr of their isolation. These cellular fractions were routinely checked for their purity by measuring marker enzyme activities and electron microscopic examination; only minimal (4 - 6%) cross contamination was noted.

1. Determination of enzyme activities

About 50 - 100 µg of the membrane protein was employed for each reaction in this study. Each enzyme reaction was linear with respect to the time and protein concentration. The results at appropriate places were analyzed statistically by the Student "t" test.

(a) $Na^+ - K^+$ ATPase

For routine estimation of $Na^+ - K^+$ stimulated ATPase activity, the fractions were incubated in a total volume of 1 ml containing 50 mM Tris-1 mM EDTA buffer, pH 7.5, 4 mM $MgCl_2$, 100 mM NaCl and 20 mM KCl in the presence or absence of ouabain (1 mM). After 5 min of pre-incubation at 37°C, the reaction was started by addition of Tris-ATP (4 mM final concentration) and the fraction was further incubated for 10 min. The reaction was stopped by the addition of 1 ml of 12% cold trichloroacetic acid, centrifuged and the Pi the clear supernatant was determined. The difference of the activities in the absence and presence of ouabain is referred to as $Na^+ - K^+$ stimulated, Mg^{2+} dependent, ouabain sensitive $Na^+ - K^+$ ATPase. The values for the enzyme activity thus obtained varied between

80 - 90% of the ATP hydrolysis due to the presence of Na^+ and K^+ in the incubation medium.

(b) Mg^{2+} ATPase

The Mg^{2+} ATPase activity was assayed in a total volume of 1 ml containing 50 mM Tris-HCl, pH 7.5, 1.25 mM Mg Cl_2 and 1.25 mM Tris-ATP. In some experiments as indicated in the text, 50 mM Tris buffer containing 1 mM EDTA, pH 7.5, 4 mM Mg Cl_2 and 4 mM Tris-ATP was used. After 5 min of pre-incubation at 37°C of the enzyme preparation in the above medium, the reaction was started by addition of ATP and the fraction was further incubated for 10 min. The reaction was stopped by the addition of 1 ml of 12% cold trichloroacetic acid, the tubes were centrifuged and the Pi in the clear supernatant was determined. The ATP hydrolysis that occurred in the absence of Mg^{2+} was subtracted in order to calculate the activity due to Mg^{2+} stimulated ATPase.

(c) Ca^{2+} ATPase

The Ca^{2+} ATPase activity was assayed in a total volume of 1 ml containing 50 mM Tris-HCl, pH 7.5, 1.25 mM Ca Cl_2 and 1.25 mM Tris-ATP. In some experiments as indicated in the text 50 mM Tris buffer containing 1 mM EDTA, pH 7.5, 4 mM Ca Cl_2 and 4 mM Tris-ATP was used. After 5 min of pre-incubation at 37°C with the enzyme in the above medium, the reaction was started by addition of ATP and the fraction was further incubated for 10 min. The reaction was stopped by the addition of 1 ml of 12% cold trichloroacetic acid, the tubes were centrifuged and Pi in the clear supernatant was determined. The ATP hydrolysis that occurred in the absence of Ca^{2+} was subtracted in order to calculate the activity due to Ca^{2+} stimulated ATPase.

J. Marker Enzyme Activities

The activities of the marker enzymes, glucose - 6 - phosphatase and cytochrome C oxidase were determined according to the methods described elsewhere (214 - 216). Acid phosphatase was estimated by determining Pi released (217) following the incubation of the membrane fraction with 25 mM β-glycerol phosphate buffered at pH 5.0 with 50 mM acetate in a final volume of 200 µl for 20 min at 37°C.

K. Determination of Calcium-Binding Activities:

Calcium binding by the sarcolemmal fraction was determined by employing the millipore filtration technique (214) in a medium containing 50 mM Tris-HCl, pH 7.0 at 37°C with a protein concentration of 0.15 to 0.20 mg/ml. The reaction was initiated by the addition of 0.1 mM $^{45}Ca\ Cl_2$ and terminated by millipore filtration. The radioactivity in the protein-free filtrates were estimated in a Packard Liquid Scintillation Spectrometer using Bray's solution.

L. Determination of Inorganic Phosphate (Pi) and Protein:

The estimation of Pi was carried out by the method of Taussky and Shorr (217). Usually 0.5 ml of the clear supernatant was employed for estimation of phosphate. Protein concentration was determined by the method of Lowry et al. (218).

IV. RESULTS

A. Characterization of Membrane Fractions:

The rat heart membrane fractions employed in this study were examined under electron microscope and these were found to be devoid of any apparent myofibrillar or nuclear contamination (Fig. 1). No intact mitochondria were seen in the sarcolemmal and microsomal fractions. The data in Table 1 indicate that mitochondrial and microsomal fractions contained very low activities of ouabain-sensitive $Na^+ - K^+$ ATPase whereas the specific activity of glucose - 6 - phosphatase in the mitochondrial and sarcolemmal fractions was markedly less than that in the microsomal fraction. On the other hand, the specific activities of acid phosphatase and cytochrome C oxidase in the sarcolemmal and microsomal fractions were not appreciable in comparison to those in the mitochondrial fraction. These marker enzyme and electron microscopic studies reveal that there is a minimal cross contamination among these membrane fractions. As reported previously (79, 115) the sarcolemmal fraction, unlike mitochondrial and microsomal fractions, did not show ATP - dependent calcium accumulation.

It can be seen from Fig. 2 that the sodium dodecyl sulphate-polyacrylamide gel electrophoresis patterns, indicating various protein bands, were different for the sarcolemmal, mitochondrial and microsomal fractions. Although the presence of some small quantities of non-membraneous proteins in the cellular fractions employed in this study can not be ruled out, it should be pointed out that the electrophoretic patterns representing different protein bands in these membrane fractions did not change upon repeated extraction with 0.6 M KCl solution. Furthermore the sarcolemmal, mitochondrial and microsomal fractions showed differences with respect to

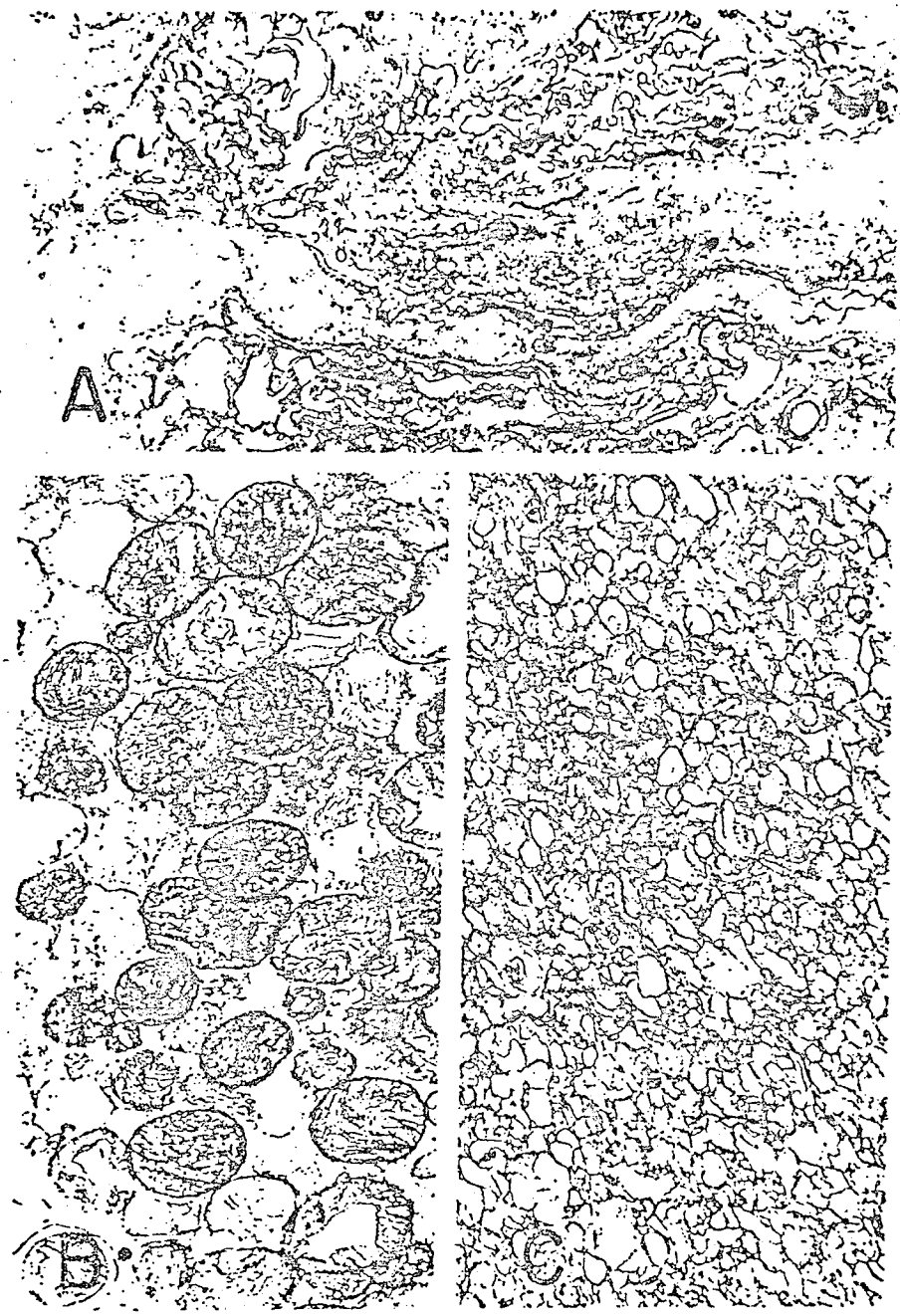

FIGURE 1 Electron micrographs of typical membrane preparation isolated from rat heart. A, Sarcolemma; B, Mitochondria; and C, Microsomes (Fragments of sarcoplasmic reticulum). (X16500).

TABLE I

Marker Enzyme Activities of Subcellular Fractions of Rat Heart

	Sarcolemma	Mitochondria	Microsomes
$Na^+ - K^+$ ATPase	12.6 ± 0.7	0.03 ± 0.02	0.04 ± 0.03
Glucose - 6 - phosphatase	0.16 ± 0.04	0.12 ± 0.03	1.56 ± 0.32
Acid phosphatase	0.05 ± 0.02	0.24 ± 0.03	0.08 ± 0.02
Cytochrome C oxidase	20 ± 5	1280 ± 40	69 ± 13

The activities of $Na^+ - K^+$ ATPase, glucose - 6 - phosphatase and acid phosphatase are expressed as μ moles Pi/mg protein per hr whereas the activity of cytochrome C oxidase is given as n moles cytochrome oxidase/ mg protein per min. Each value is a mean ± S.E. of six experiments.

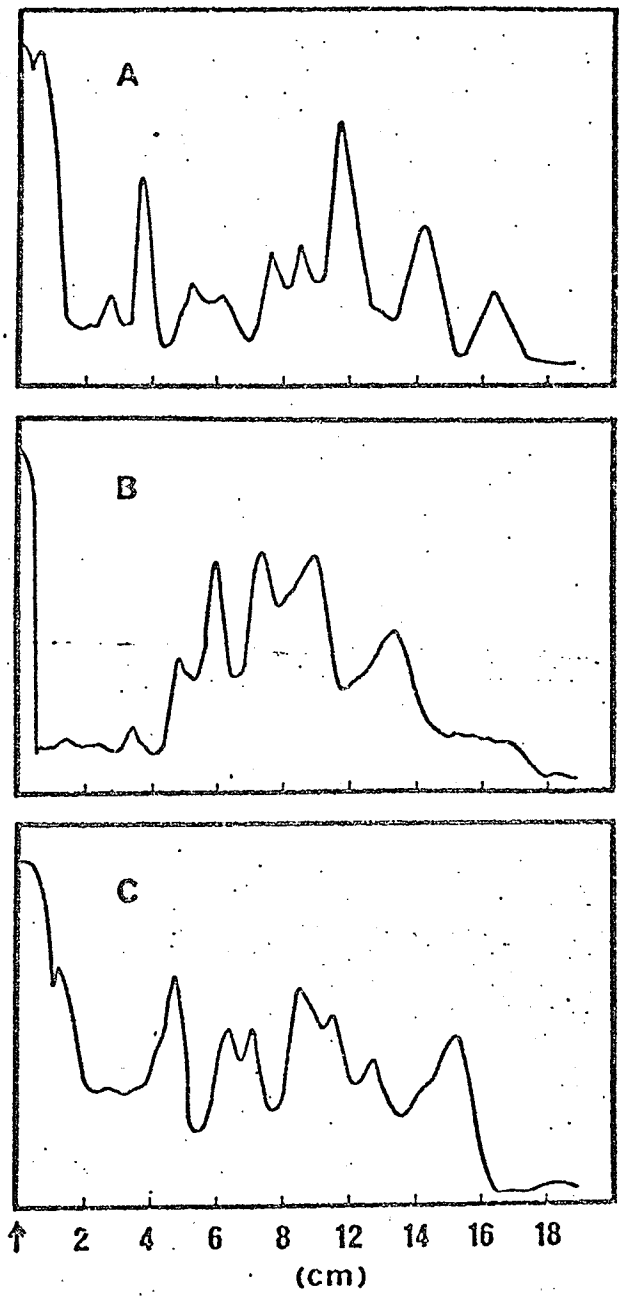

FIGURE 2 Sodium docecyl sulfate gel scans of subcellular fractions of rat heart. A, Sarcolemma; B, Mitochondria; and C, Microsomes. The abscissa represents the distance (arbitrary units in cm) moved by different bands from the point of application (arrow) of the membrane fractions.

their phospholipid composition (Table II). For example, the microsomal fraction had higher total phospholipid contents as well as proportion of phosphatidyl choline. On the other hand, the sarcolemmal fraction had higher proportions of lysophosphatidyl choline and phosphatidyl inositol and phosphatidyl serine but the proportion of phosphatidyl ethanolamine was lower in comparison to the mitochondrial fraction. These studies concerning differences in the physical and chemical make-ups further suggest that these fractions are enriched with organelles derived from different origins.

B. Ca^{2+}/Mg^{2+} ATPase Activities of Sarcolemmal, Mitochondrial and Microsomal Fractions:

In one set of experiments, the ATP hydrolyzing activities of different membrane fractions were studied in the presence of various concentrations of Ca^{2+} or Mg^{2+}. It should be noted that these experiments were carried out in the presence of 1 mM EDTA. The results in Fig. 3 show that these cations at 4 - 8 mM concentrations stimulated the ATP hydrolysis by sarcolemma, mitochondria and microsomes maximally. The Ca^{2+} ATPase activities of mitochondria, at 4 to 16 mM cation concentrations were higher than sarcolemma but lower than microsomes. The Ca^{2+} ATPase activity was higher than the Mg^{2+} ATPase activity in the case of sarcolemma only. Other divalent cations such as Mn^{2+}, Co^{2+}, Ni^{2+}, Ba^{2+} were also capable of stimulating ATP hydrolysis by these membrane fractions and the order of their potency was different for each fraction (Table III). The maximal ATP hydrolyzing activities of microsomal and mitochondrial fractions in the presence of Ca^{2+} or Mg^{2+} were observed at pH 8.0 - 8.5 whereas that of the sarcolemmal fraction was attained at pH 7.5 - 8.0 (Fig. 4).

In another series of experiments the effects of some calcium

TABLE II

Phospholipid Composition of Subcellular Fractions of Rat Heart.

	Sarcolemma	Mitochondria	Microsomes
A. Total phospholipids:	0.22 ± 0.02	0.29 ± 0.03	0.52 ± 0.03
B. Phospholipid contents:			
Cardiolipin + phosphatidic acid	19.5 ± 1.2	20.5 ± 1.8	18.4 ± 1.6
Phosphatidyl ethanolamine	26.1 ± 0.7	34.6 ± 1.7	27.8 ± 0.8
Phosphatidyl inositol + phosphatidyl serine	14.8 ± 0.6	6.3 ± 1.5	9.2 ± 1.0
Phosphatidyl choline	26.3 ± 2.3	29.8 ± 1.4	40.2 ± 1.8
Sphingomyelin	9.3 ± 0.9	7.0 ± 0.6	4.1 ± 0.3
Lysophosphatidyl choline	3.8 ± 0.3	1.6 ± 0.2	0.2 ± 0.1

The values for total phospholipids were expressed as mg/mg membrane protein whereas those for the individual phospholipid contents were expressed as % of the total phospholipids. Each value is a mean ± S.E. of 4 experiments.

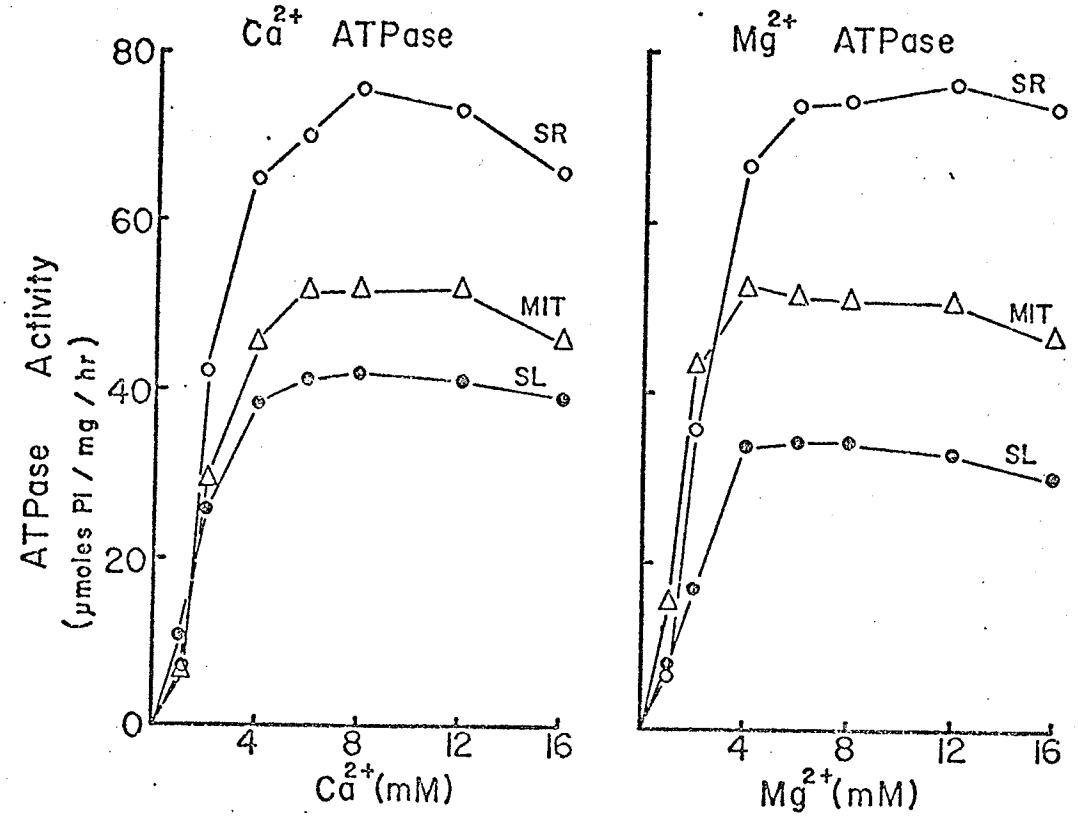

FIGURE 3 Effect of different concentrations of Ca^{2+} or Mg^{2+} on the ATP hydrolyzing activities of rat heart sarcolemma (SL), microsomes (SR) and mitochondria (MIT). The incubation medium contained 50 mM Tris-HCl, pH 7.5, 1 mM EDTA and 4 mM ATP. Each value is an average of 6 experiments.

TABLE III

Relative Rates of ATP Hydrolysis by Heart Membranes in the Presence of Different Divalent Cations

Cations	ATP hydrolysis		
	Sarcolemma	Microsomes	Mitochondria
Ca^{2+}	100	100	100
Mg^{2+}	66.8 ± 4.6	107.1 ± 2.1	106.0 ± 3.2
Mn^{2+}	65.4 ± 4.1	60.2 ± 3.6	162.7 ± 6.7
Co^{2+}	57.6 ± 3.7	44.9 ± 3.3	121.7 ± 4.5
Ni^{2+}	14.9 ± 2.3	5.1 ± 0.7	23.5 ± 2.4
Ba^{2+}	26.3 ± 3.4	0.8 ± 0.2	18.3 ± 1.1
Sr^{2+}	14.7 ± 1.3	0.0 ± 0.0	3.0 ± 0.6

Membranes were incubated in the presence of 4 mM of different cations in a medium containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA and 4 mM ATP. The ATP hydrolysis with each cation is expressed as % of that due to Ca^{2+}. Each value is a mean ± S.E. of 3 experiments.

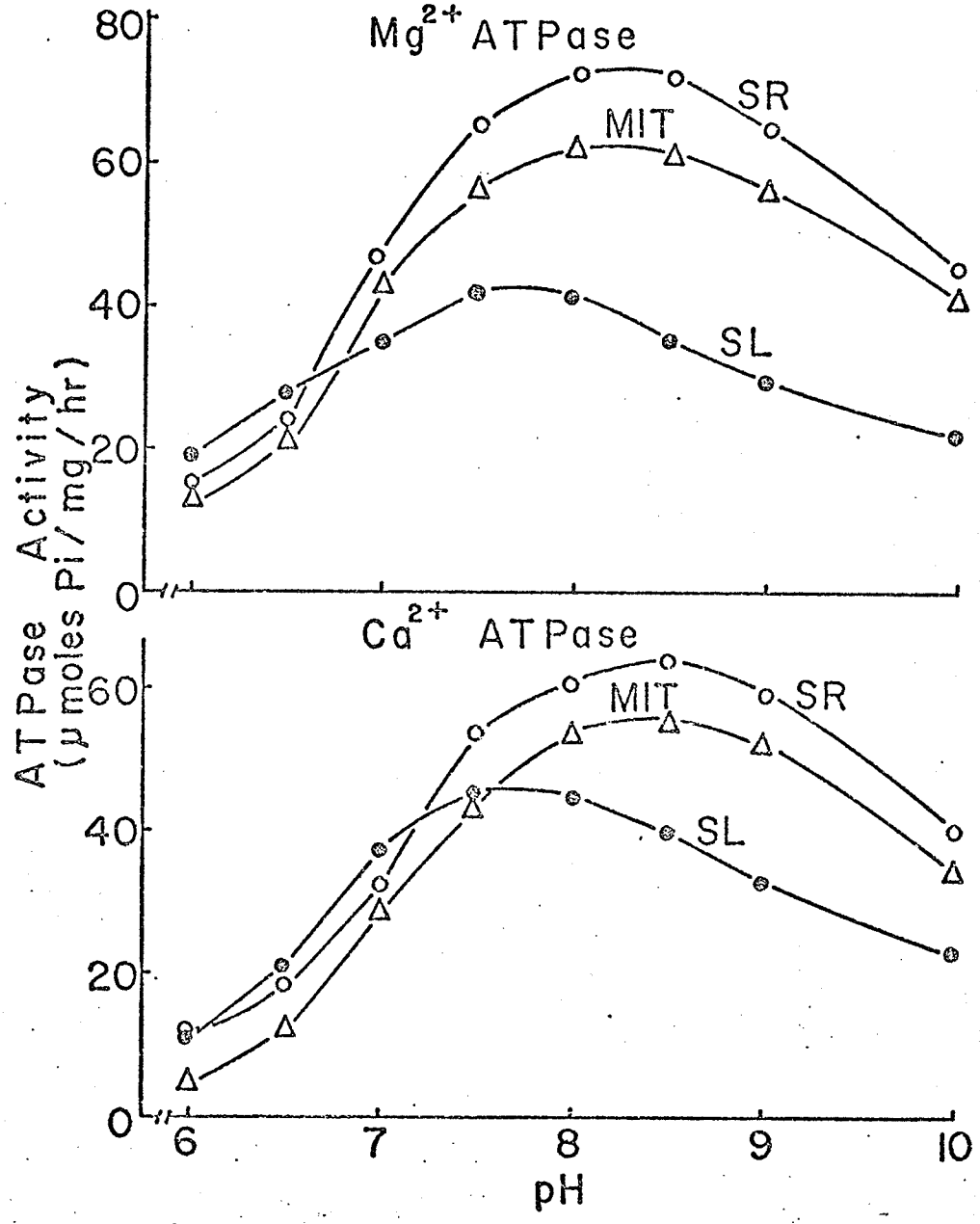

FIGURE 4 pH profiles of the ATP hydrolyzing activities of rat heart sarcolemma (SL), microsomes (SR), and mitochondria (MIT) in the presence of 4 mM Ca^{2+} or Mg^{2+}. The experimental conditions were same as those described for Fig. 3. Each value is an average of 5 experiments.

transport inhibitors on the ATP hydrolyzing activities of sarcolemma, mitochondria and microsomes were tested in the presence of 4 mM Ca ATP or Mg ATP and 1 mM EDTA. The results are given in Table IV. A significant stimulatory effect of DNP on Ca^{2+} ATPase or Mg^{2+} ATPase activities was seen in mitochondria only. On the other hand, sodium azide decreased mitochondrial Ca^{2+} ATPase and Mg^{2+} ATPase activities but Ca^{2+} ATPase was less sensitive than Mg^{2+} ATPase. No significant effect of sodium azide was seen on microsomal ATPase activities while sarcolemmal Mg^{2+} ATPase, but not Ca^{2+} ATPase was significantly inhibited by sodium azide. Although ruthenium red inhibited all the membrane ATPase activities, the microsomal ATPases were more sensitive than sarcolemmal but less sensitive than mitochondria. It should be also noted that the Ca^{2+} ATPase activities of these membranes were inhibited by ruthenium red to a greater extent in comparison to the Mg^{2+} ATPase activities. It was interesting to find that lanthanum at concentrations of 100 μM or less had an inhibitory effect on the sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase activities without significantly affecting the ATPase activities of mitochondria and microsomes. NaF was found to decrease sarcolemmal and mitochondrial ATPase activities but the inhibition of Ca^{2+} ATPase was greater than that of Mg^{2+} ATPase activities. NaF also decreased the microsomal Ca^{2+} ATPase whereas Mg^{2+} ATPase was not affected by this agent at 1 - 8 mM concentrations. It should be noted that DNP, sodium azide and ruthenium red have been shown to inhibit mitochondrial calcium transport (214, 219, 220) whereas lanthanum is considered to displace calcium from the cell membrane (221) and NaF has been shown to affect microsomal calcium transport (222).

The actions of some inhibitors of the sulfhydryl, carboxyl and amino groups on membrane ATPase activities were also studied under the

TABLE IV

Effects of Some Inhibitors on the ATP Hydrolyzing Activities of Rat Heart Membranes in the Presence of 4 mM Ca^{2+} or Mg^{2+}.

	ATPase activity					
	Ca^{2+} ATPase			Mg^{2+} ATPase		
Additions	Sarcolemma	Mitochondria	Microsomes	Sarcolemma	Mitochondria	Microsomes
Dinitrophenol:						
0.1 mM	100 ± 1	114 ± 3	100 ± 2	99 ± 1	109 ± 2	101 ± 2
0.4 mM	105 ± 3	126 ± 3	101 ± 1	105 ± 3	120 ± 3	104 ± 3
Sodium azide:						
1 mM	94 ± 3	36 ± 6	95 ± 2	80 ± 3	15 ± 3	92 ± 4
5 mM	93 ± 4	35 ± 5	96 ± 2	66 ± 4	13 ± 2	91 ± 4
Ruthenium red:						
30 µg/ml	85 ± 2	52 ± 5	76 ± 4	93 ± 2	58 ± 5	94 ± 3
150 µg/ml	66 ± 4	33 ± 3	44 ± 6	74 ± 4	38 ± 4	60 ± 4
Lanthanum:						
10 µM	85 ± 3	96 ± 3	100 ± 1	66 ± 5	96 ± 3	95 ± 2
100 µM	71 ± 4	91 ± 4	99 ± 1	63 ± 4	105 ± 3	95 ± 3
Sodium fluoride:						
1 mM	101 ± 1	105 ± 3	97 ± 2	103 ± 2	95 ± 2	103 ± 2
8 mM	56 ± 6	30 ± 5	50 ± 6	75 ± 4	80 ± 3	99 ± 1

The ATPase activities are expressed as % of the respective control value in the absence of these agents. The experimental conditions were the same as those for Table III. Each value is a mean ± S.E. of 4 experiments.

above mentioned experimental conditions and the results are shown in Table V. PCMB was found to inhibit all the membrane Ca^{2+} ATPase and Mg^{2+} ATPase activities except that it had a biphasic effect on the sarcolemmal Mg^{2+} ATPase activity. Cysteine(1 mM), which had no effect of its own was found to protect against the inhibitory action of PCMB on the membrane ATPases. It was also observed that microsomal ATPase activities were inhibited by PCMB to a greater extent than the mitochondrial enzyme activities. Iodoacetate was found to stimulate the sarcolemmal Mg^{2+} ATPase activity slightly at low concentrations, however, this agent inhibited all membrane ATPase activities in high concentrations. The degree of inhibition of Ca^{2+} ATPase activities in these membranes was somewhat greater than that of Mg^{2+} ATPase activities. The results shown also indicate that iodoacetamide increased mitochondrial Ca^{2+} ATPase and decreased microsomal Ca^{2+} ATPase and Mg^{2+} ATPase activities only. On the other hand, maleic anhydride was found to decrease sarcolemmal Ca^{2+} ATPase and increase mitochondrial Mg^{2+} ATPase activities only. Agents such as PCMB, iodoacetate and iodoacetamide are believed to inhibit sulfhydryl groups whereas maleic anhydride and carbodiimide are considered to react with amino and carboxyl groups respectively (103, 223).

C. Comparison of Ca^{2+} ATPase and Mg^{2+} ATPase Activities of Heart Sarcolemma:

The results described in the previous section indicated some differences in the activation patterns of ATP hydrolysis by heart sarcolemma in the presence of Ca^{2+} and Mg^{2+}. Further information on this aspect was obtained by studying the properties of the heart sarcolemmal ATP hydrolysis in the presence of Ca^{2+} or Mg^{2+}. In one series of experiments, heart sarcolemma was incubated in the presence of 4 mM Ca^{2+} or Mg^{2+} and the ATP

TABLE V

Effects of Various Inhibitors on the ATP Hydrolyzing Activities of Rat Heart Membranes in the Presence of 4 mM Ca^{2+} or Mg^{2+}.

	ATPase activity					
	Ca^{2+} ATPase			Mg^{2+} ATPase		
Additions	Sarcolemma	Mitochondria	Microsomes	Sarcolemma	Mitochondria	Microsomes
p-chloromercuribenzoate:						
2.5 µM	103 ± 2	104 ± 2	90 ± 2	133 ± 4	103 ± 2	85 ± 3
15 µM	54 ± 3	82 ± 3	53 ± 4	75 ± 3	81 ± 3	63 ± 4
Iodoacetate:						
1 mM	93 ± 4	93 ± 2	83 ± 3	108 ± 2	98 ± 2	98 ± 3
8 mM	70 ± 4	25 ± 5	50 ± 6	75 ± 3	86 ± 3	70 ± 3
Iodoacetamide:						
0.1-1 mM	99 ± 2	104 ± 2	98 ± 2	110 ± 3	101 ± 2	95 ± 1
2.0-5 mM	104 ± 3	115 ± 2	97 ± 3	124 ± 4	102 ± 2	89 ± 2
Carbodiimide:						
0.1-1 mM	98 ± 2	95 ± 1	94 ± 1	102 ± 2	101 ± 2	90 ± 2
2.0-5 mM	97 ± 2	89 ± 2	91 ± 2	101 ± 1	100 ± 1	85 ± 2
Maleic anhydide:						
0.1-1 mM	92 ± 1	99 ± 2	99 ± 3	100 ± 1	109 ± 2	100 ± 1
2.0-5 mM	86 ± 3	96 ± 2	99 ± 2	102 ± 2	115 ± 3	103 ± 2

The ATPase activities are expressed as % of the respective control value in the absence of these agents. The experimental value is a mean ± S.E. of 4 experiments.

hydrolysis was measured by employing different concentrations of ATP. Lineweaver-Burk analysis of these data showed low and high affinity sites for both Ca^{2+} and Mg^{2+} ATPases (Fig. 5). The K_m value for the low affinity sites for Ca^{2+} ATPase and Mg^{2+} ATPase was 318 μM whereas the V_{max} values were 33 and 25 μmoles Pi/mg/hr respectively. The V_{max} values for the high affinity sites for Ca^{2+} ATPase and Mg^{2+} ATPase varied between 15.1 - 15.6 μmoles Pi/mg/hr whereas the K_m values were 81 and 106 μM respectively.

In order to test the substrate specificity for the sarcolemmal ATP hydrolyzing enzyme systems, the abilities of heart sarcolemma to hydrolyze different nucleotides were studied in the presence of Ca^{2+} or Mg^{2+}. The results in Table VI did not indicate any substrate specificity; however, the order of nucleotide hydrolysis in the presence of Ca^{2+} was different from that in the presence of Mg^{2+}. The effects of ADP and Pi, the products of ATP hydrolysis, were also tested on the sarcolemmal ATPase activities. Both ADP and Pi were found to inhibit the sarcolemmal ATP hydrolysis; Mg^{2+} ATPase was more sensitive to ADP and less sensitive to Pi inhibition in comparison to Ca^{2+} ATPase (Table VII). Furthermore, storage of sarcolemma at 0 - 2°C for different days was associated with a greater increase in ATP hydrolysis in the presence of Ca^{2+} in comparison to that in the presence of Mg^{2+} (Table VIII). Although freezing and thawing of heart sarcolemma decreased the ATP hydrolyzing activities, the depression in the presence of Ca^{2+} was not appreciably different from that in the presence of Mg^{2+} (Table IX). Likewise, the ATPase activities in the presence of Ca^{2+} and Mg^{2+} decreased upon heating the heart sarcolemma at 40 - 75°C for 5 min; the enzyme activities were not detectable in membrane preparations heated at 75°C (Table X).

The effects of different monovalent and divalent cations were

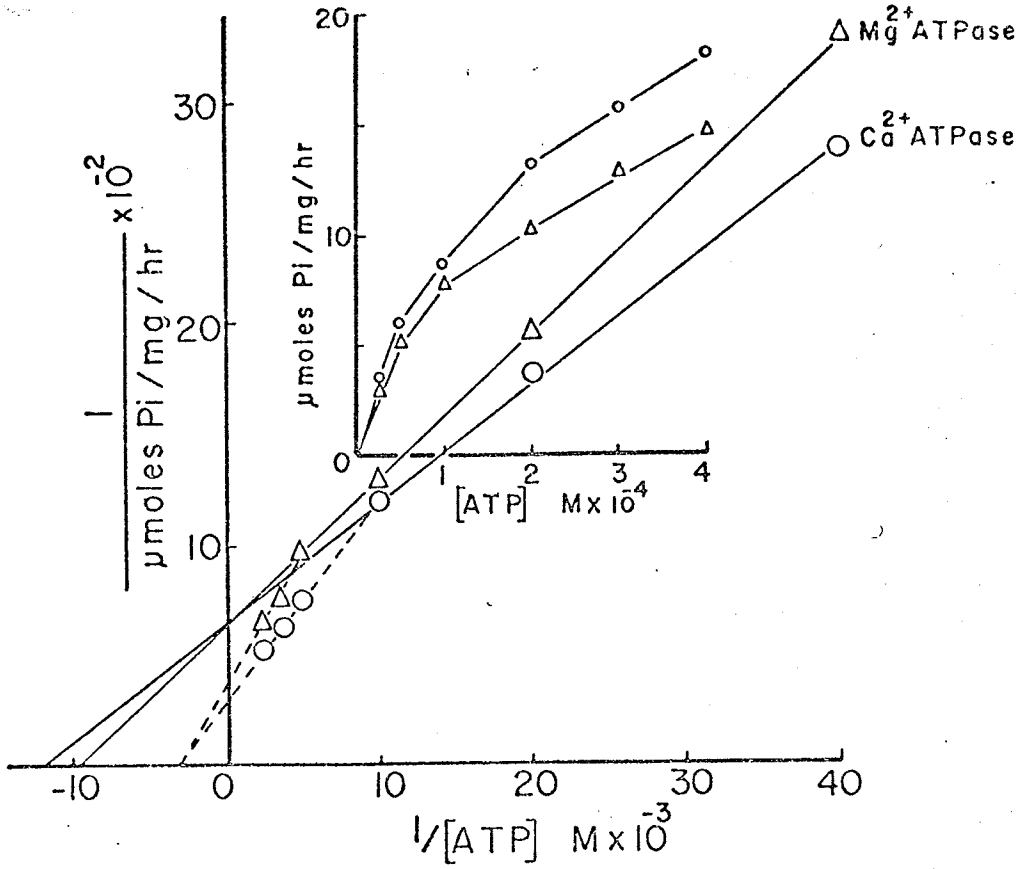

FIGURE 5 Effect of different concentrations of ATP on ATP hydrolysis by heart sarcolemma due to the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+}. The experimental conditions were the same as those described for Fig. 3. The values are typical of 3 experiments.

TABLE VI

Hydrolysis of Different Nucleotides by Heart Sarcolemma in the Presence of Ca^{2+} or Mg^{2+}.

Nucleotides	4 mM Ca^{2+}	4 mM Mg^{2+}
ATP	35.2 ± 2.6	29.8 ± 2.2
GTP	18.2 ± 1.7	27.3 ± 1.5
UTP	29.3 ± 2.3	23.6 ± 1.9
CTP	26.6 ± 1.6	19.3 ± 2.7
ADP	12.4 ± 1.7	11.9 ± 1.3
AMP	2.5 ± 0.48	4.8 ± 0.36

Hydrolysis of each nucleotide is expressed as μmoles Pi released/mg protein/hr. The concentration of each nucleotide was 4 mM and the incubation medium contained 50 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Each value is a mean ± S.E. from 3 different preparations.

TABLE VII

Product Inhibition of Heart Sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase Activities

Additions	4 mM Ca^{2+}	4 mM Mg^{2+}
Control	35.6 ± 2.1	25.9 ± 1.9
ADP (mM):		
1	23.4 ± 2.2	15.1 ± 1.1
2	21.5 ± 1.6	9.6 ± 0.7
3	17.9 ± 1.4	9.2 ± 0.8
4	15.3 ± 0.9	9.1 ± 0.5
Pi (mM):		
1	32.4 ± 2.0	23.7 ± 1.7
2	19.4 ± 1.5	20.7 ± 2.0
3	17.2 ± 1.6	18.5 ± 1.4
4	16.0 ± 1.3	17.7 ± 1.2

ATPase activity is expressed as μmoles Pi released/mg protein/hr. The experimental conditions are the same as those for Table III. The values are a mean ± S.E. from 3 different preparations.

TABLE VIII

Effect of Aging on Heart Sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase Activities

Storage (Hours)	4 mM Ca^{2+}	4 mM Mg^{2+}
24	110 ± 3.7	103 ± 1.4
48	132 ± 4.3	112 ± 2.1
120	144 ± 6.2	123 ± 3.9
216	187 ± 8.4	131 ± 3.7

ATPase activity is expressed as % of the control. The control value for the fresh preparations for Ca^{2+} and Mg^{2+} were 36.4 ± 2.7 and 27.6 ± 2.1 µmoles Pi/mg protein/hr respectively. The preparations were stored at 0 - 2°C for the desired time and their activity determined at 37°C. The experimental conditions are the same as those for Table III. The values are a mean ± S.E. from 4 different preparations.

TABLE IX

Effect of Freezing and Thawing on Heart Sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase Activities

Number of freezing and thawing	4 mM Ca^{2+}	4 mM Mg^{2+}
0	37.9	28.2
1	32.5	24.8
2	28.2	20.5
3	29.8	19.4
4	25.3	18.0

ATPase activity is expressed as μmoles Pi released/mg protein/hr. The experimental conditions were the same as those for Table III. Each value is an average from 3 different preparations.

TABLE X

Effect of Heating Heart Sarcolemma at Different Temperatures on Ca^{2+} ATPase and Mg^{2+} ATPase Activities

Temperature (°C)	4 mM Ca^{2+}	4 mM Mg^{2+}
Control	38.5	26.9
40	33.1	23.8
45	23.9	16.0
50	20.3	15.9
55	14.9	11.4
60	2.1	1.6
75	0.0	0.0

ATPase activity is expressed as μmoles Pi released/mg protein/hr. Preparations were heated at the desired temperature for 5 minutes and then the reaction was carried out as described in "Methods" at 37°C. Unheated preparations served as control. The experimental conditions were the same as those for Table III. Each value is an average from 3 different preparations.

also tested on the ATP hydrolyzing activities of heart sarcolemma in the presence of Ca^{2+} or Mg^{2+}. It can be seen from the data in Table XI that monovalent cations such as Na^{+}, K^{+}, NH^{+}, Li^{+} and Cs^{+} had no appreciable effect on the Ca^{2+} or Mg^{2+} ATPase activities. On the other hand, divalent cations such as Ni^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+} decreased both Ca^{2+}/Mg^{2+} ATPase activities whereas Ba^{2+} and Sr^{2+} were ineffective (Fig. 6 and 7). The inhibitory effect of Mn^{2+}, unlike that of Ni^{2+}, Co^{2+} and Cu^{2+}, on Ca^{2+} ATPase was greater than that on Mg^{2+} ATPase.

The influence of different agents known to affect calcium fluxes in myocardium was also tested on the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase and the results are shown in Table XII. Verapamil, epinephrine and cyclic AMP were found to have no effect on Ca^{2+}/Mg^{2+} ATPases whereas a calcium ionophore (A-23187) increased the Ca^{2+} ATPase activity slightly but significantly ($P < 0.05$). When Ca^{2+}/Mg^{2+} ATPase activities were monitored under optimal conditions by employing 4 mM Ca ATP or Mg ATP in the presence of 1 mM EDTA, these agents including ionophore (A-23187) were ineffective (data not shown). Since effects of cyclic AMP are now well known to be mediated through the protein kinase mediated phosphorylation, sarcolemmal ATP hydrolysis was measured after treatments with cyclic AMP-protein kinase. It can be seen from Figs. 8 and 9 that ATP hydrolysis in the presence of different concentrations of Ca^{2+} or Mg^{2+} by the phosphorylated membranes was higher than that by the control preparations. It should also be noted that the increased ability of the phosphorylated heart sarcolemma to hydrolyze ATP in the presence of Ca^{2+} or Mg^{2+} was associated with an increase in V_{max} values without any changes in the K_a values. The K_a values for the control sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase were 0.57 - 0.68 and 0.71 - 0.88 mM whereas the V_{max} values were about 35 and 25 μmoles Pi/mg/hr respectively. The V_{max}

TABLE XI

Effect of Monovalent Ions on Heart Sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase Activities

	4 mM Ca^{2+}	4 mM Mg^{2+}
Control	33.0	26.5
Na^{+}	34.1	27.5
K^{+}	34.0	26.7
NH_4^{+}	33.6	23.9
Li^{+}	33.6	24.0
Cs^{+}	31.6	24.8

ATPase activity is expressed as μmoles Pi released/mg protein/hr. Concentration of each monovalent ion was varied from 5 to 100 mM. The experimental conditions were the same as those for Table III. The values are an average from 4 different preparations.

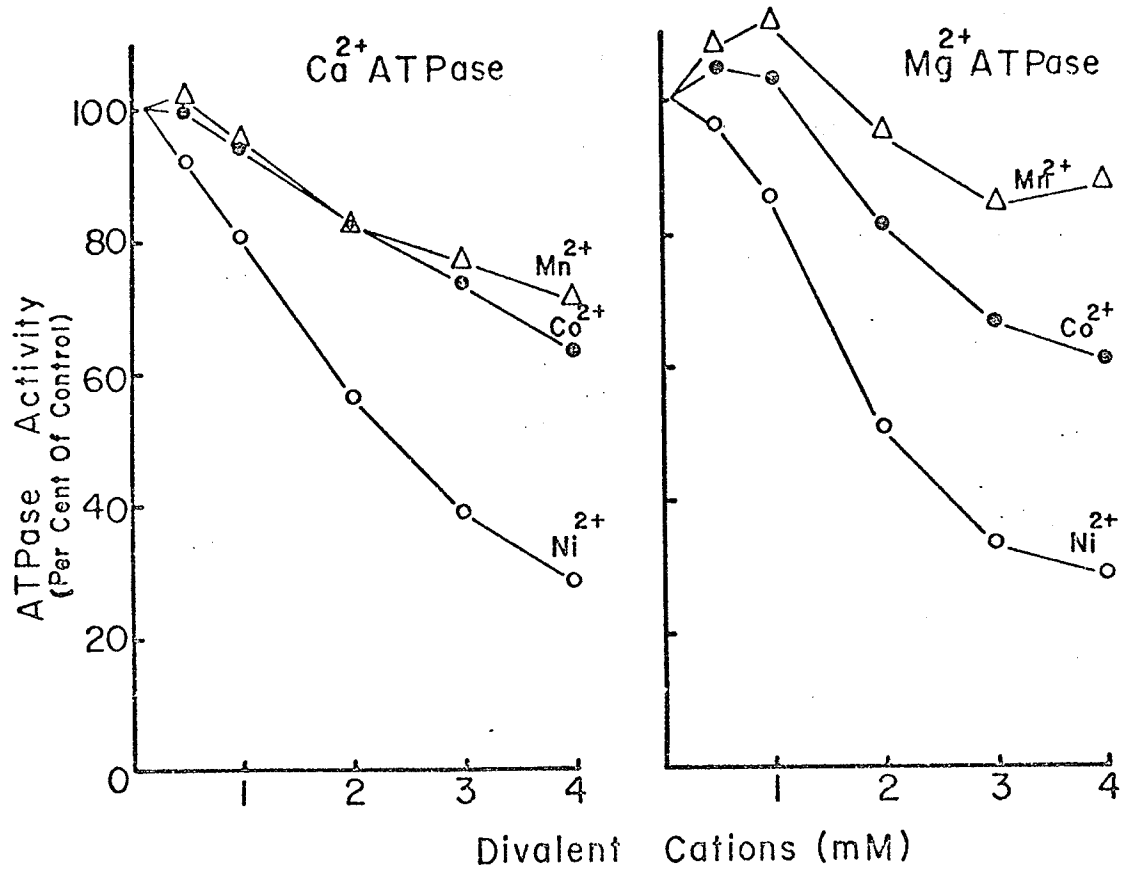

FIGURE 6 Effect of different concentrations of Mn^{2+}, Co^{2+} and Ni^{2+} on ATP hydrolysis by heart sarcolemma due to the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+}. The experimental conditions were the same as those described for Fig. 3. The control values for ATP hydrolysis in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+} were 37.5 $\pm$ 2.7 and 26.5 $\pm$ 2.5 µmoles Pi/mg protein/hr respectively. Each value is an average from 3 different preparations.

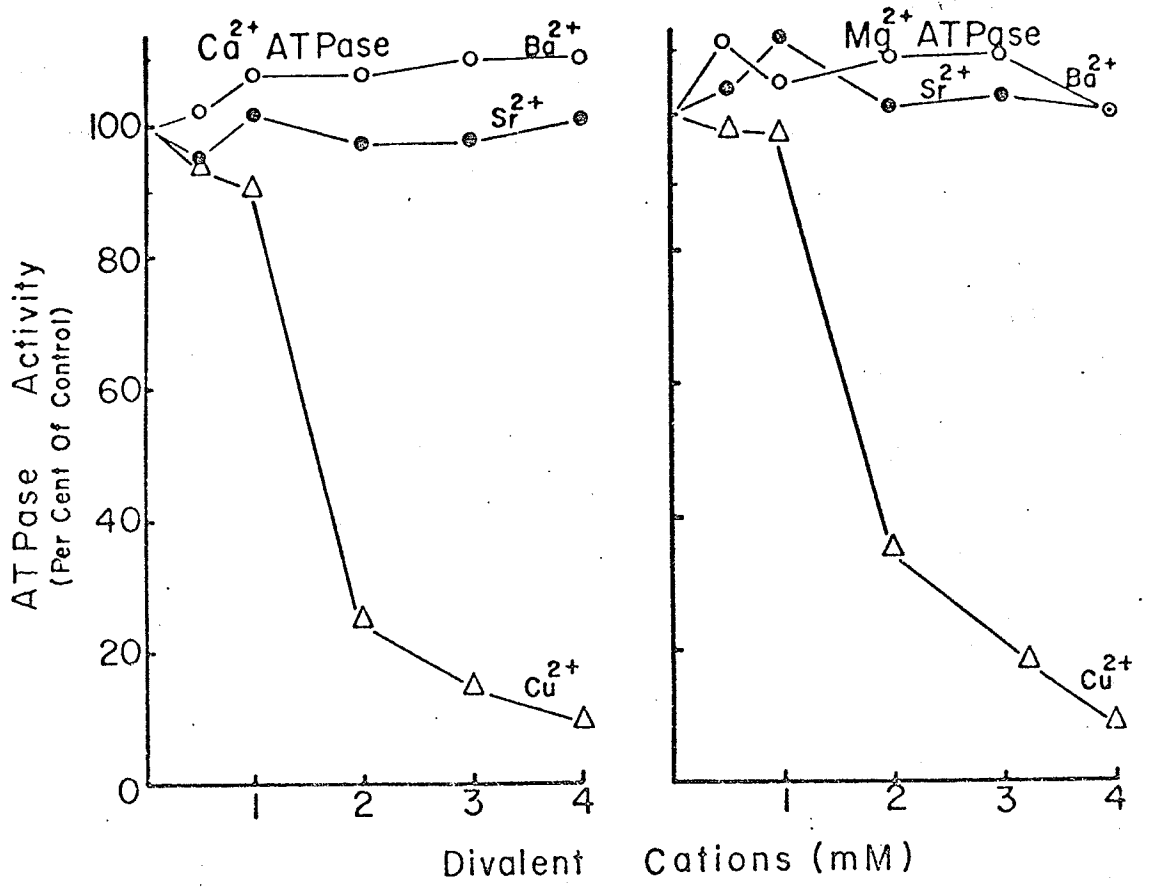

FIGURE 7 Effect of different concentrations of Ba^{2+}, Sr^{2+} and Cu^{2+} on ATP hydrolysis by heart sarcolemma due to the presence of 4 mM Ca^{2+} or Mg^{2+}. The experimental conditions were the same as those described for Fig. 3. The control value for ATP hydrolysis in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+} were 37.0 $\pm$ 2.8 and 27.2 $\pm$ 2.1 µ moles Pi/mg protein/hr respectively. Each value is an average from 3 different preparations.

TABLE XII

Effect of Different Agents Known to Influence Calcium Fluxes in Myocardium on Heart Sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase Activities

Additions	1.25 mM Ca^{2+}	1.25 mM Mg^{2+}
Verapamil (0.5 - 5 µM)	98 ± 2.7	104 ± 3.4
A-23187 ionophore (1 - 8 µg)	112 ± 3.6	94 ± 3.1
Epinephrine (25 - 100 µM)	102 ± 1.7	103 ± 2.2
Cyclic AMP (10^{-6} - 10^{-5}M)	101 ± 1.2	103 ± 2.0

ATPase activity is expressed as % of control. The control values for Ca^{2+} and Mg^{2+} were 7.8 ± 0.5 and 7.4 ± 0.3 µmoles Pi/mg protein/hr respectively; ATP concentration used in the medium was 1.25 mM. Each value is a mean ± S.E. of 3 - 4 different preparations.

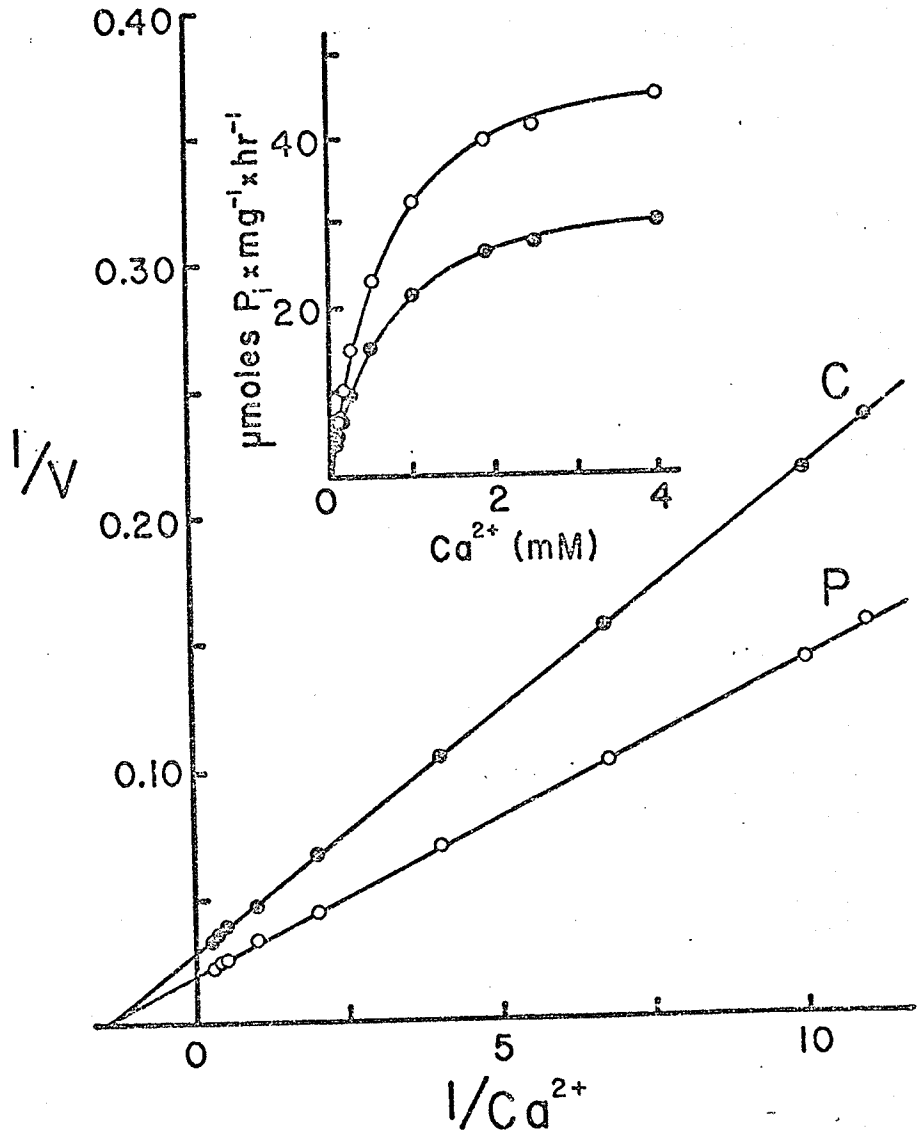

FIGURE 8 Effect of cyclic AMP-protein kinase mediated phosphorylation on ATP-hydrolysis by heart sarcolemma in the presence of different concentrations of Ca^{2+}. C, Control; and P, Phosphorylated preparation. The incubation medium contained 50 mM Tris-HCl, pH 7.5 and 4 mM ATP. Each value is an average from 3 different preparations.

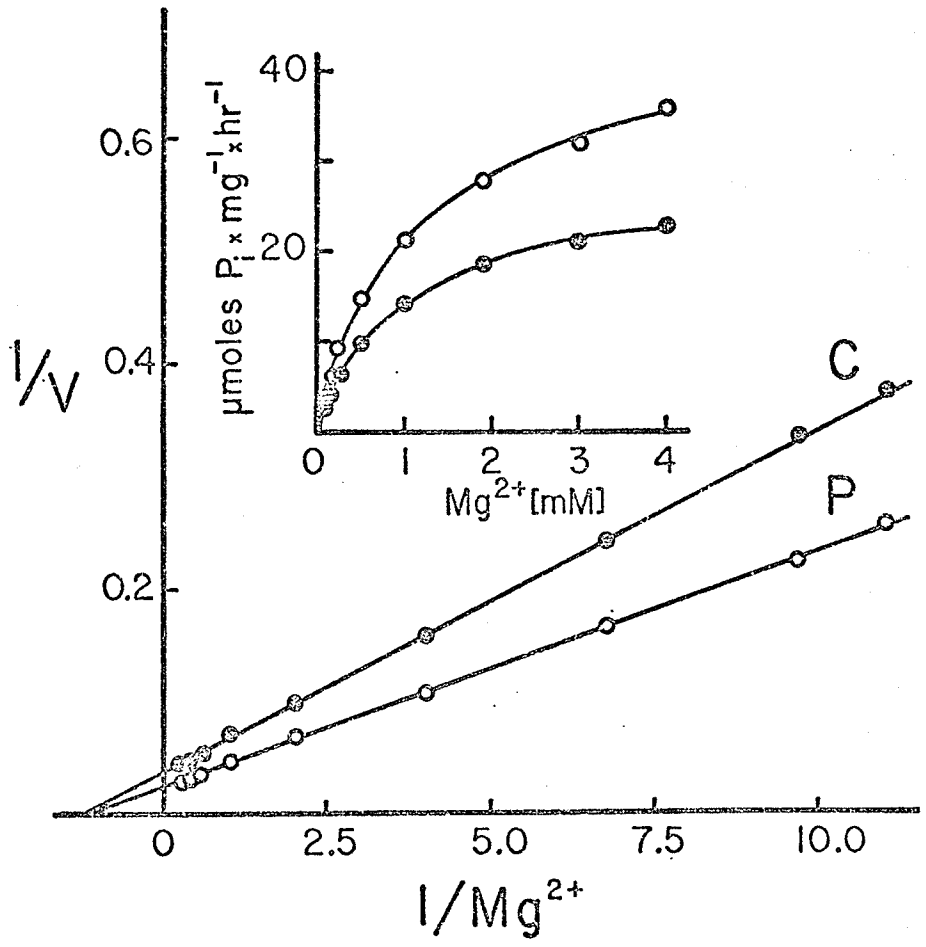

FIGURE 9 Effect of cyclic AMP-protein kinase mediated phosphorylation on ATP hydrolysis by heart sarcolemma in the presence of different concentrations of Mg^{2+}. C, Control; and P, phosphorylated preparation. The incubation medium contained 50 mM Tris-HCl, pH 7.5, and 4 mM ATP. Each value is an average from 3 different preparations.

values for the phosphorylated sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase were about 44 and 35 µmoles Pi/mg/hr respectively.

D. Effects of Phospholipase Treatments on Heart Sarcolemmal Structure and Ca^{2+}/Mg^{2+} ATPase Activity:

In one set of experiments, heart sarcolemma was treated with phospholipases A, C and D for 10 min, the preparations were thoroughly washed and examined electron microscopically either after fixing and embedding (Fig. 10) or after negative staining (Fig. 11). In both cases varying degrees of morphological changes in the membrane structure were apparent upon phospholipase treatments. Furthermore, the effectiveness of the phospholipase A (0.25 mg/mg protein) and phospholipase C and D (0.50 mg/mg protein) treatments for 10 min was evident from the reduced calcium binding ability of the treated preparations. The calcium binding values for the control, phospholipase A, phospholipase C and phospholipase D treated heart sarcolemma were 64.4, 18.2, 40.6 and 27.5 n moles Ca^{2+}/ mg protein/5 min respectively.

The effects of different concentrations of phospholipases A, C and D treatments on the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase activities were studied and the results are reported in Figs. 12 and 13. Both phospholipase A and D treatments did not produce any appreciable depressant effect on Ca^{2+} ATPase whereas phospholipase C treatment produced a slight but definite biphasic effect. On the other hand, Mg^{2+} ATPase activity was markedly decreased in phospholipase A and C treated sarcolemma whereas phospholipase D treatment in low concentrations produced a slight but significant ($P < 0.05$) increase. Prolonging the time of phospholipase A treatment from 10 to 60 min did not produce any further changes in the sarcolemmal Ca^{2+} or Mg^{2+} ATPase activity. In contrast to Ca^{2+} ATPase,

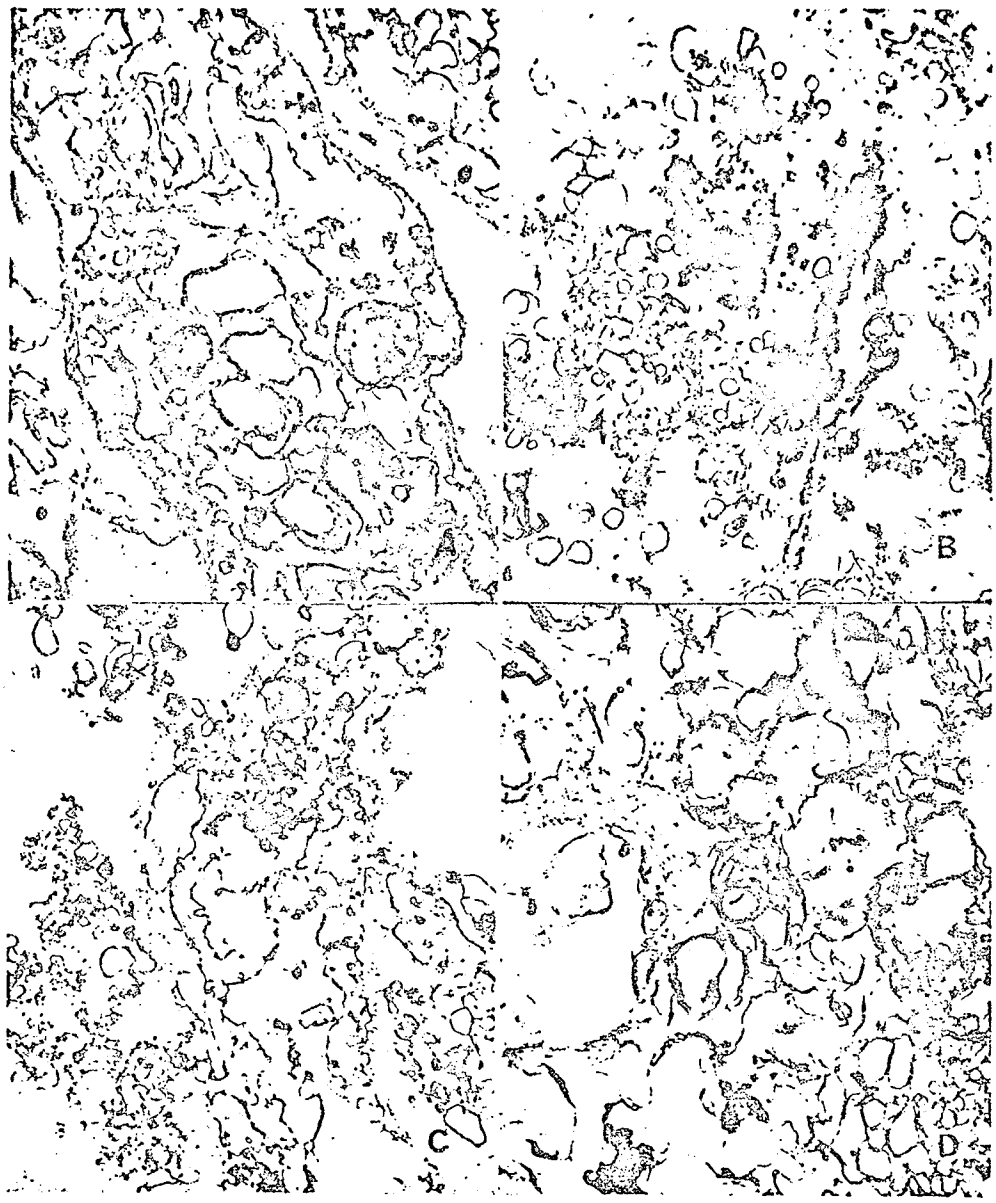

FIGURE 10 Electron micrographs of heart sarcolemma with or without phospholipase treatment. A, Control; B, phospholipase A treatment (0.25 mg/mg membrane protein) for 10 min; C, phospholipase C treatment (0.50 mg/mg membrane protein) for 10 min; and D, phospholipase D treatment (0.50 mg/mg of membrane protein) for 10 min. (X26,730).

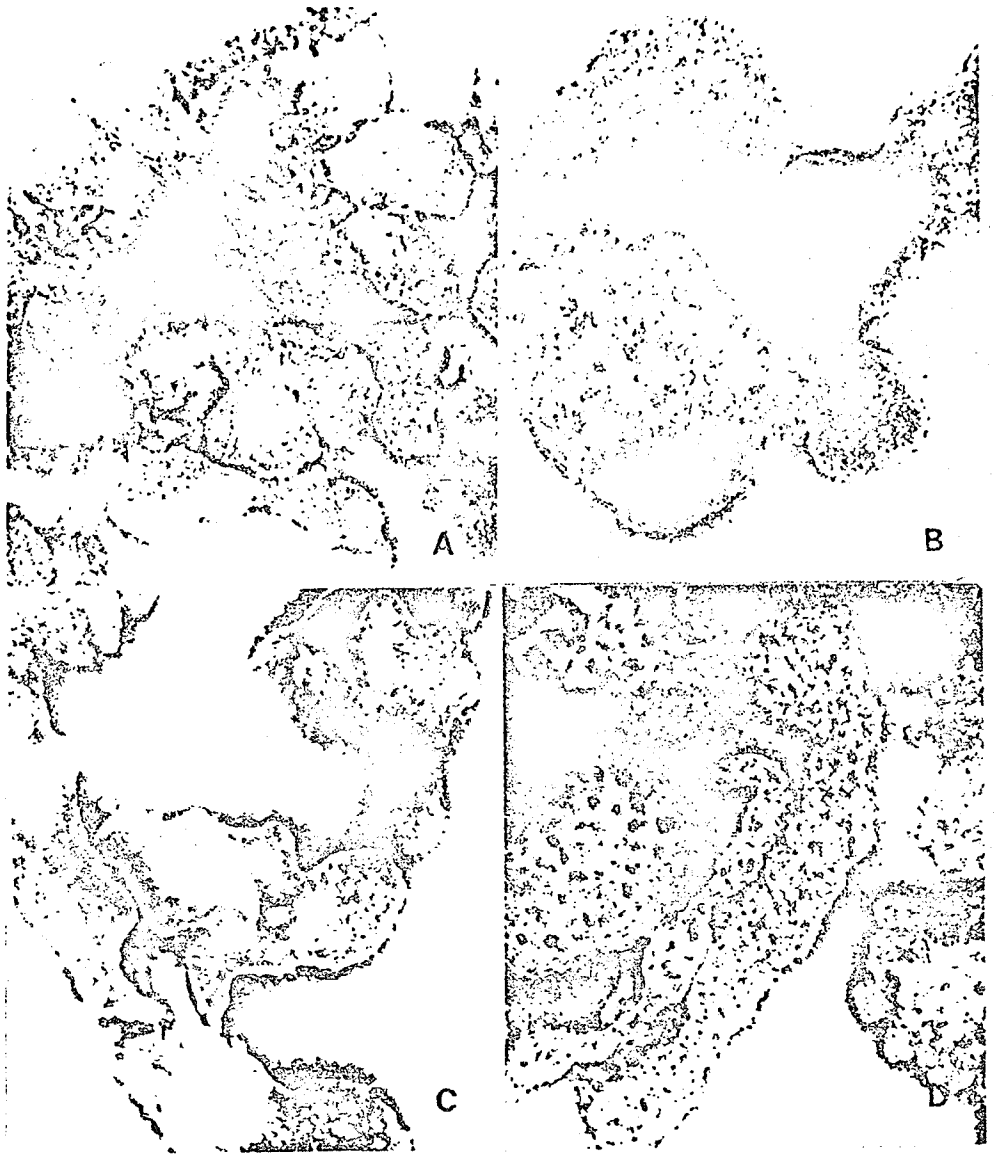

FIGURE 11 Negative staining of heart sarcolemma with or without phospholipase treatment. A; Control; B, phospholipase A treatment (0.25 mg/mg membrane protein) for 10 min; C, phospholipase C treatment (0.50 mg/mg of membrane protein) for 10 min; and D, phospholipase D treatment (0.50 mg/mg membrane protein) for 10 min. (X95,700).

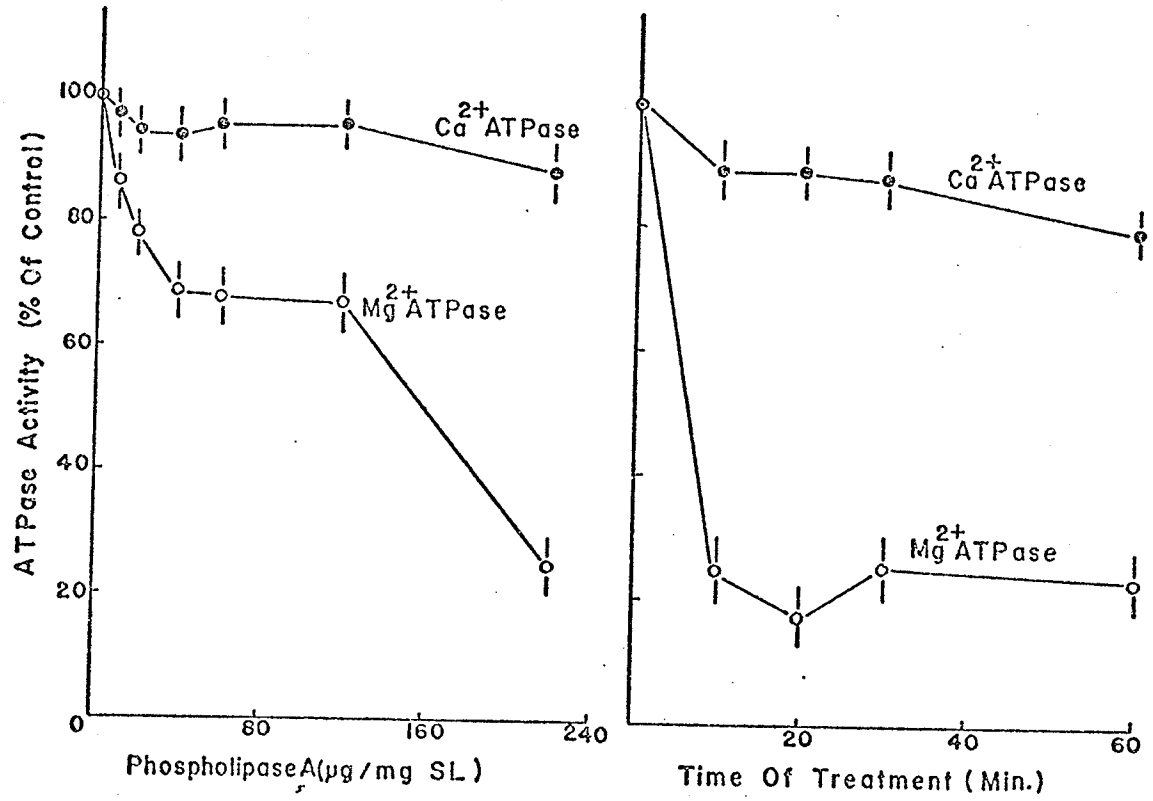

FIGURE 12 Effect of different concentrations and different times of phospholipase A treatment of heart sarcolemma on ATP hydrolysis in the presence of 1.25 mM Ca^{2+} or Mg^{2+}. The incubation medium contained 50 mM Tris-HCl, pH 7.5, and 1.25 mM Tris-ATP. Each value is a mean $\pm$ S.E. of 3 experiments.

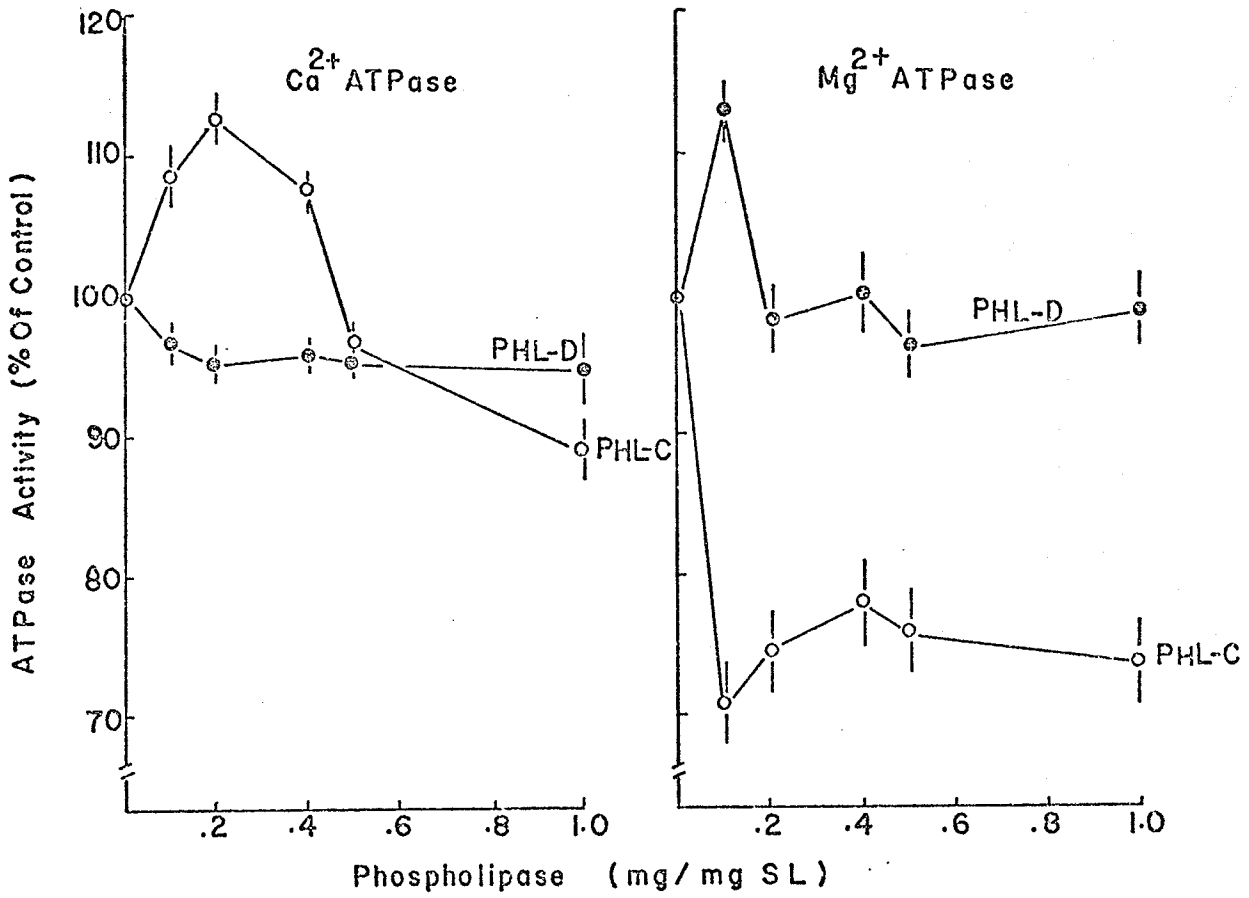

FIGURE 13 Effects of different concentrations of phospholipase C (PHL-C) and phospholipase D (PHL-D) treatments of heart sarcolemma on ATP hydrolysis in the presence of 1.25 mM Ca^{2+} or Mg^{2+}. The experimental conditions were the same as those for Fig. 12. Each value is a mean $\pm$ S.E. of 3 experiments.

Mg^{2+} ATPase decreased markedly upon increasing the time of phospholipase C or D treatment from 10 to 60 min (Fig. 14). These data indicate that Ca^{2+} ATPase was less sensitive to phospholipase treatments in comparison to the Mg^{2+} ATPase and that phospholipase A was more active than phospholipases C and D. This pattern was also noted when the ATPase activities was monitored in the presence of different concentrations of Ca^{2+} or Mg^{2+} in the incubation medium. Lineweaver-Burke plot of the data obtained by employing different concentrations of Mg^{2+} revealed that the inhibitory effects of phospholipases A, C and D treatments were associated with an increase in K_a values for Mg^{2+} from 0.87 to 1.5 mM (Fig. 15). It should also be noted that the V_{max} value for the phospholipase A treated membranes, unlike that of the phospholipase C treated preparations was decreased whereas that for the phospholipase D treated preparations was increased. This may indicate some differences in the mechanisms of actions of these phospholipases on heart sarcolemma.

Since the membrane preparations employed in this study are vesicular in shape, it is possible that some sites concerned with Ca^{2+}/ Mg^{2+} ATPase activity are not available for the phospholipase action. This possibility was tested by employing membrane preparations which were made leaky after a mild treatment with detergent such as deoxycholate. The results shown in Table XIII indicate different degrees of depression in the Ca^{2+}/Mg^{2+} ATPase activities of the deoxycholate-treated sarcolemma upon treatments with different phospholipases; however, Mg^{2+} ATPase was still more sensitive than Ca^{2+} ATPase to the phospholipase action. It should be pointed out that the addition of 1 mM fat-free albumin in the incubation medium for the ATPase activity did not abolish the depression in the Ca^{2+}/Mg^{2+}

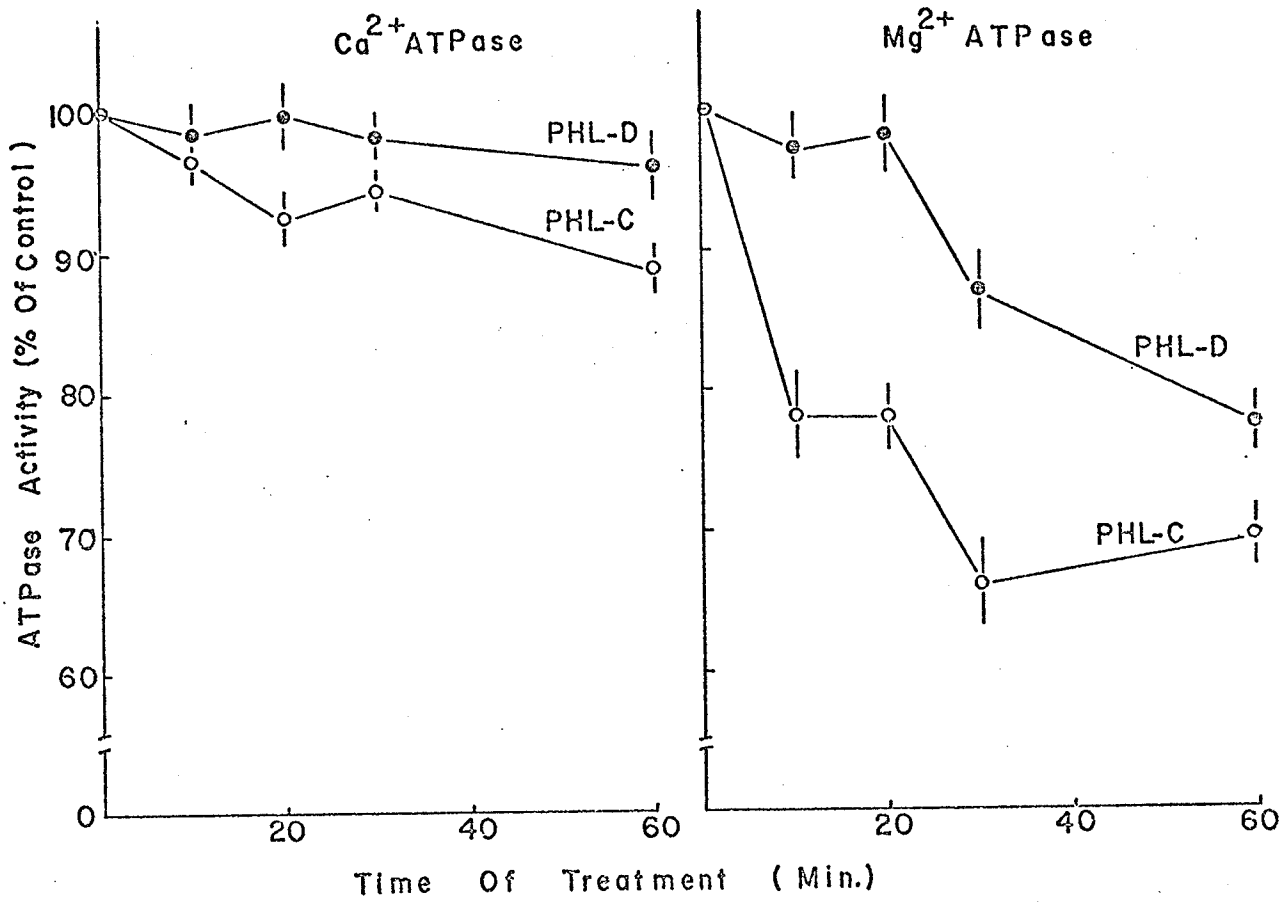

FIGURE 14 Effects of different times of treatments of heart sarcolemma with phospholipase C (PHL-C) and phospholipase D (PHL-D) on ATP hydrolysis in the presence of 1.25 mM Ca^{2+} or Mg^{2+}. The experimental conditions were the same as those for Fig. 12. Each value is a mean $\pm$ S.E. of 3 experiments.

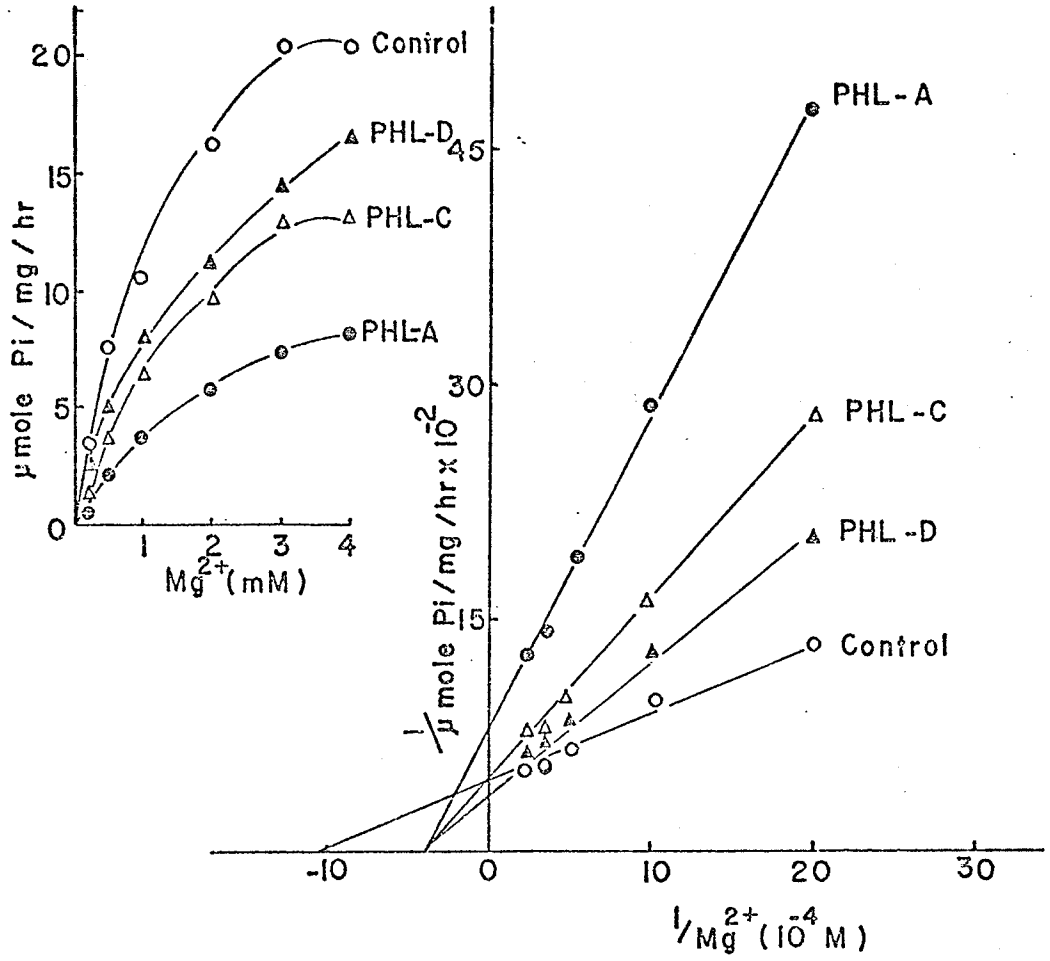

FIGURE 15 Effects of phospholipase A (PHL-A), phospholipase C (PHL-C) and phospholipase D (PHL-D) treatments of heart sarcolemma on ATP hydrolysis in the presence of different concentrations of Mg^{2+}. The concentration of PHL-A used was 250 µg/mg of membrane protein; PHL-C and PHL-D were used at a concentration of 0.5 mg/mg of membrane protein. The incubation medium contained 50 mM Tris-HCl, pH 7.5 and 4 mM Tris-ATP. The results are typical of 4 experiments.

TABLE XIII

Effect of Phospholipases A, C and D on Deoxycholate Treated Sarcolemmal Ca^{2+}/Mg^{2+} ATPase Activities

	ATPase activities (μ moles Pi released/mg/hr)	
	1.25 mM Ca^{2+}	1.25 mM Mg^{2+}
Control	29.9 ± 1.4	19.8 ± 0.7
Phospholipase A (0.25 mg/mg protein)	18.3 ± 0.9	4.1 ± 0.2
Phospholipase C (0.5 mg/mg protein)	21.5 ± 0.8	16.2 ± 0.6
Phospholipase D (0.5 mg/mg protein)	25.1 ± 1.2	15.1 ± 0.3

ATP concentration used in the medium was 1.25 mM. Each value is a mean ± S.E. of 4 different preparations. The sarcolemma was treated with deoxycholate at a concentration of 0.2 mg/mg membrane protein for 10 min at a room temperature, centrifuged, and thoroughly washed twice with 1 mM Tris buffer pH 7.0. This preparation was further treated with different phospholipases for 10 min at a room temperature, washed thoroughly and employed for ATPase determination.

ATPase activities seen in the phospholipases treated membranes. The possibility that the observed decrease in Ca^{2+}/Mg^{2+} ATPase activities in phospholipase-treated preparations due to release of free fatty acids was further ruled out because different saturated and unsaturated fatty acids were found to exert no effect on the sarcolemmal Ca^{2+}/Mg^{2+} ATPase activity (Table XIV). The depressed Mg^{2+} ATPase activity of the phospholipase A treated membranes was also not restored when these preparations were reconstituted by the addition of different concentrations of synthetic phospholipids such as lecithin, lysolecithin or phosphatidic acid (Table XV).

E. Effects of Trypsin on Heart Sarcolemmal Structure and Ca^{2+}/Mg^{2+} ATPase Activity:

The effectiveness of trypsin treatment on heart sarcolemma was tested by examining the trypsin-treated membrane electron microscopically after fixing and embedding (Fig. 16) as well as after negative staining (Fig. 17). These studies indicated dramatic morphological alterations in the membrane appearance due to trypsin treatment. The electrophoretic patterns of the trypsin-treated preparations were also found to be different from that of the control membranes (Fig. 18). This indicated changes in the protein composition of the trypsin-treated heart sarcolemma.

In one series of experiments, the influence of trypsin on the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase activities was tested by incubating membranes with trypsin and measuring ATP hydrolysis with trypsin and measuring ATP hydrolysis in its presence. It should be mentioned that trypsin in the concentrations employed in this study did not interfere with Pi measurements. Increasing the concentration of trypsin in the

TABLE XIV

Effect of Saturated and Unsaturated Fatty Acids on Ca^{2+}/Mg^{2+} ATPase Activities of Heart Sarcolemma

	ATPase activities (μ moles Pi released/mg/hr)	
	1.25 mM Ca^{2+}	1.25 mM Mg^{2+}
Control	31.6 ± 2.7	22.4 ± 1.8
Lauric Acid	32.8 ± 2.5	22.5 ± 1.8
Capric Acid	33.0 ± 2.7	22.3 ± 1.5
Palmitic Acid	31.1 ± 2.3	21.8 ± 1.4
Stearic Acid	30.6 ± 2.9	22.4 ± 1.6
Oleic Acid	29.9 ± 2.9	20.7 ± 1.7
Linoleic Acid	30.1 ± 2.8	20.3 ± 1.8
Arachidonic Acid	28.3 ± 2.6	19.7 ± 1.7

Each value is a mean ± S.E. of 3 different preparations. ATP concentration used was 1.25 mM whereas the concentrations of fatty acids were in the range of 10^{-8} to 10^{-5} M.

TABLE XV

The Effect of Synthetic Phospholipids on Ca^{2+}/Mg^{2+} ATPase of the Phospholipase A Treated Heart Sarcolemma

Addition	ATPase activities (μmoles/Pi released/mg/hr)	
	Ca^{2+} ATPase	Mg^{2+} ATPase
- -	26.0 ± 1.8	4.0 ± 0.5
Lecithin (.015 - 1.5 mg/mg protein)	26.6 ± 1.9	3.2 ± 0.7
Lysolecithin (.015 - 1.5 mg/mg protein)	26.1 ± 1.6	2.9 ± 0.6
Phosphatidic Acid (.015 - 1.5 mg/mg protein)	27.3 ± 1.8	3.2 ± 0.7

The concentrations of Ca^{2+} or Mg^{2+} and ATP were 1.25 mM. The sarcolemma was treated with phospholipase A (250 μg/mg membrane protein) for 10 min, washed thoroughly and then the effects of phospholipids were tested. Each value is a mean ± S.E. of 4 different experiments.

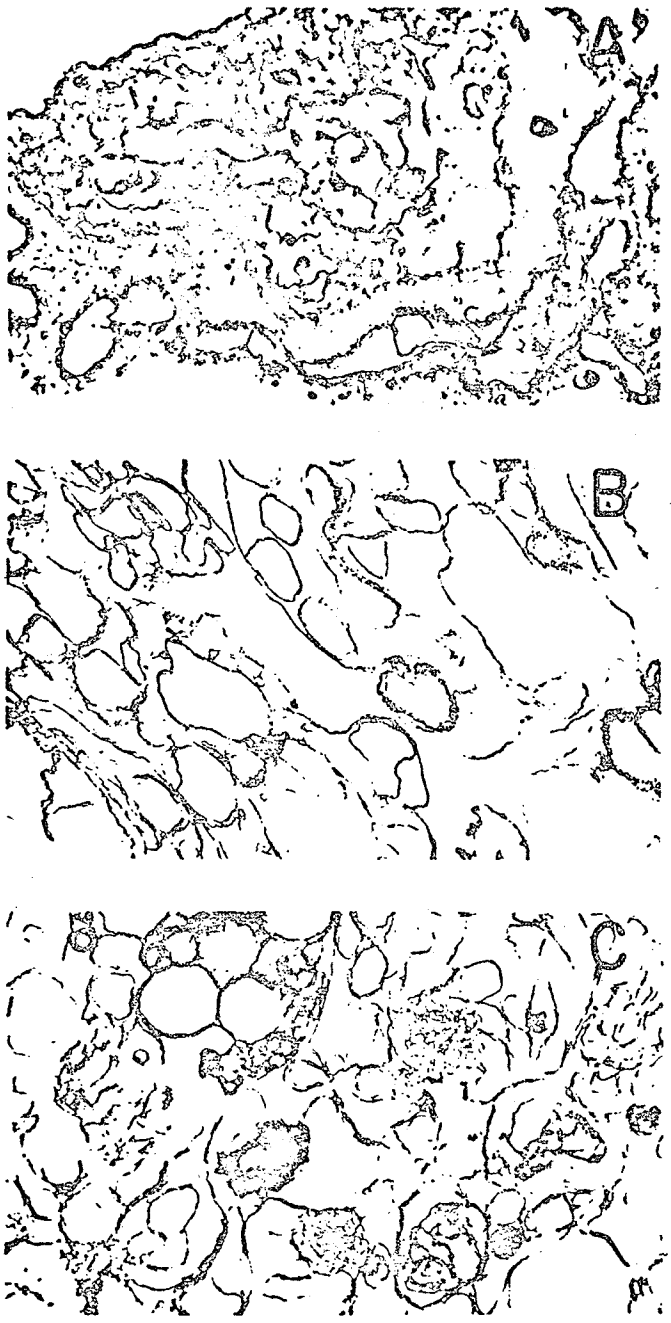

FIGURE 16 Electron micrographs of heart sarcolemma with or without trypsin treatment. A, Control; B, Trypsin (100 µg/mg membrane protein) treatment for 10 min; and C, Trypsin (100 µg/mg membrane protein) treatment for 30 min. (X26,730).

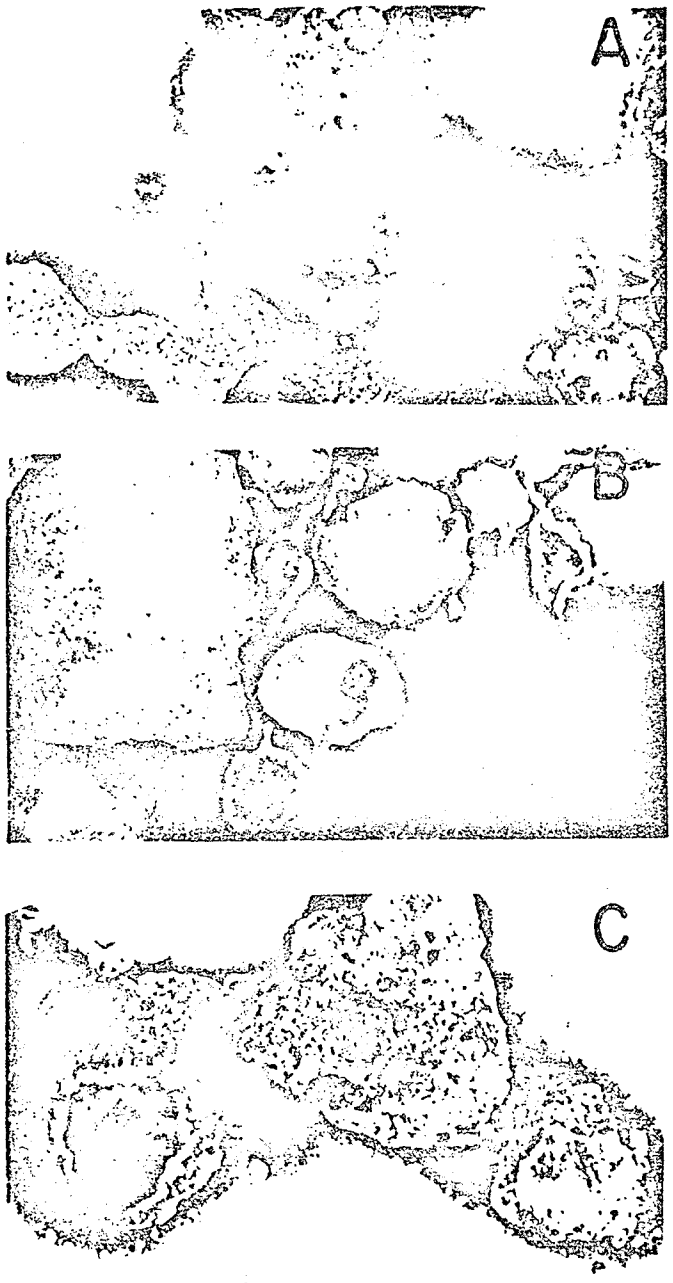

FIGURE 17 Negative staining of heart sarcolemma with or without trypsin treatment. A, Control; B, Trypsin (100 µg/mg membrane protein) treatment for 10 min; and C, Trypsin (100 µg/mg membrane protein) treatment for 30 min. (X95,700).

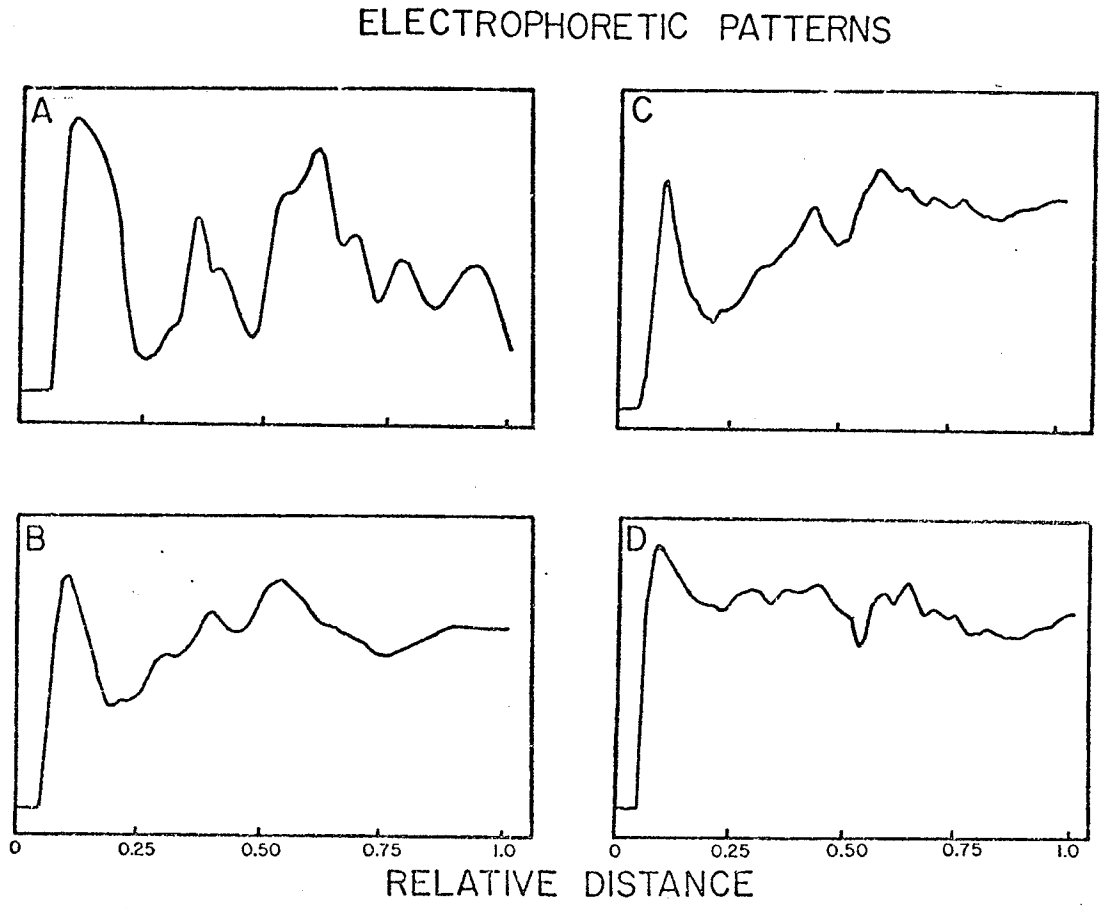

FIGURE 18 Electrophoretic patterns of heart sarcolemma with or without trypsin treatment. A, Control; B, Trypsin (100 µg/mg membrane protein) treatment for 10 min; C, Trypsin (300 µg/mg membrane protein) treatment for 10 min; and D, Trypsin (300 µg/mg membrane protein) treatment for 30 min. 0.1 mg of control and trypsin treated preparations was used per gel.

incubation medium was found to increase the Ca^{2+} ATPase activity without significantly ($P > 0.05$) affecting the Mg^{2+} ATPase activity; Fig. 19, maximal stimulation of Ca^{2+} ATPase was seen at 50 - 100 µg/mg sarcolemmal protein concentrations of trypsin. Low concentrations of trypsin (100 µg/mg membrane protein) stimulated the Ca^{2+} ATPase activity within 1 to 20 min of preincubation without any action on the Mg^{2+} ATPase (Fig. 20). On the other hand, high concentrations of trypsin (2 mg/mg membrane protein) produced a biphasic effect on Ca^{2+} ATPase and had a slight depressant effect on Mg^{2+} ATPase (Fig. 20).

The increase in heart sarcolemmal Ca^{2+} ATPase activity due to the presence of trypsin in the incubation medium was observed when different concentrations of Ca^{2+} were employed for ATPase measurements (Fig. 21). Lineweaver-Burk analysis of the data indicates that trypsin lowered the K_a value from 0.59 to 0.45 mM and increased the V_{max} value from 37 to 69 µmoles Pi/mg membrane protein/hr for Ca^{2+} ATPase. On the other hand, Mg^{2+} ATPase activity at different concentrations of Mg^{2+} was not affected by the presence of trypsin (data not shown). The possibility regarding the inability of trypsin to reach the Mg^{2+} ATPase sites in the membrane preparation was tested by employing leaky preparations obtained by mild treatments with detergents such as deoxycholate and lubrol. It was interesting to find that different concentrations of trypsin increased the Ca^{2+} ATPase activity and decreased the Mg^{2+} ATPase activity in these detergent-treated preparations (Table XVI).

In order to gain some information regarding the release of Ca^{2+}/Mg^{2+} ATPase in the supernatant upon trypsin treatment, the distribution of the total and specific enzyme activities was examined by incubating

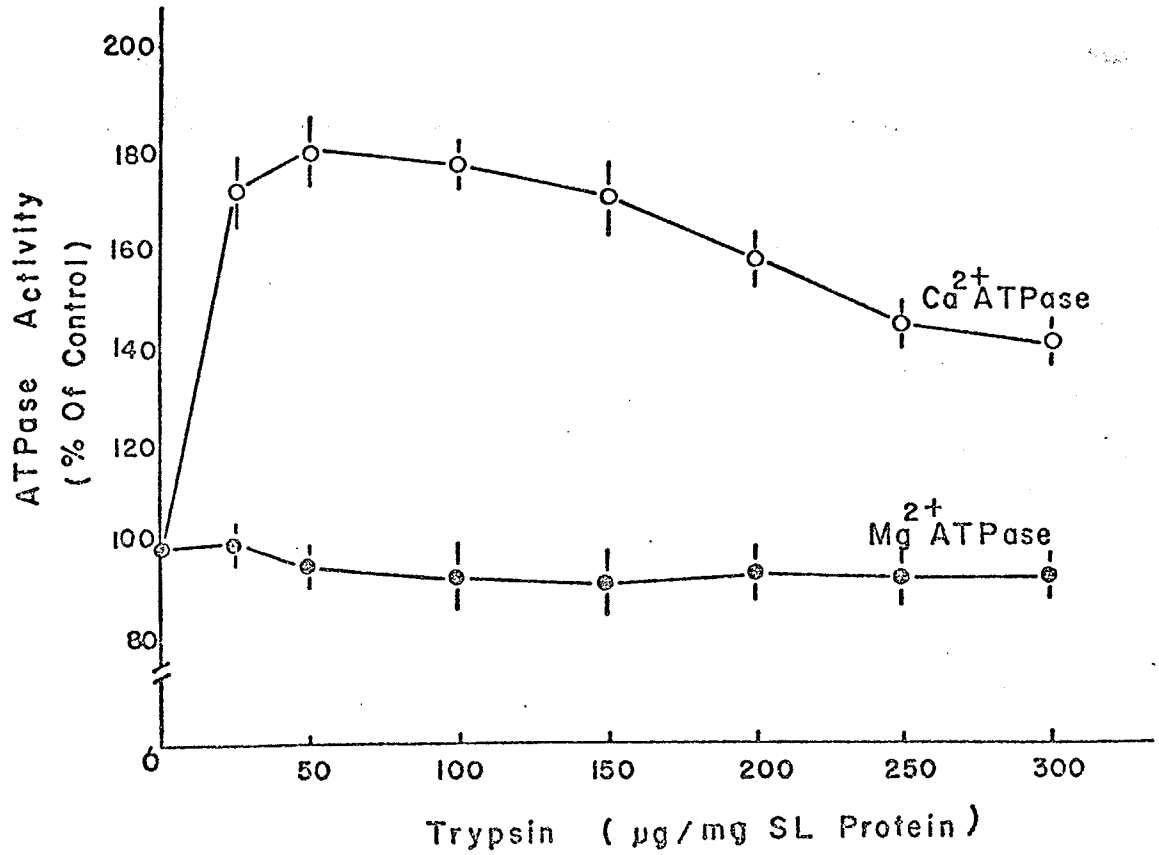

FIGURE 19 Effect of different concentrations of trypsin on ATP hydrolysis by heart sarcolemma in the presence of 1.25 mM Ca^{2+} or Mg^{2+}. ATP hydrolysis was measured in the presence of trypsin in a medium containing 50 mM Tris-HCl, pH 7.5, and 1.25 mM Tris-ATP. Preincubation time of sarcolemma with trypsin was 5 min. Each value is a mean $\pm$ S.E. of 4 experiments.

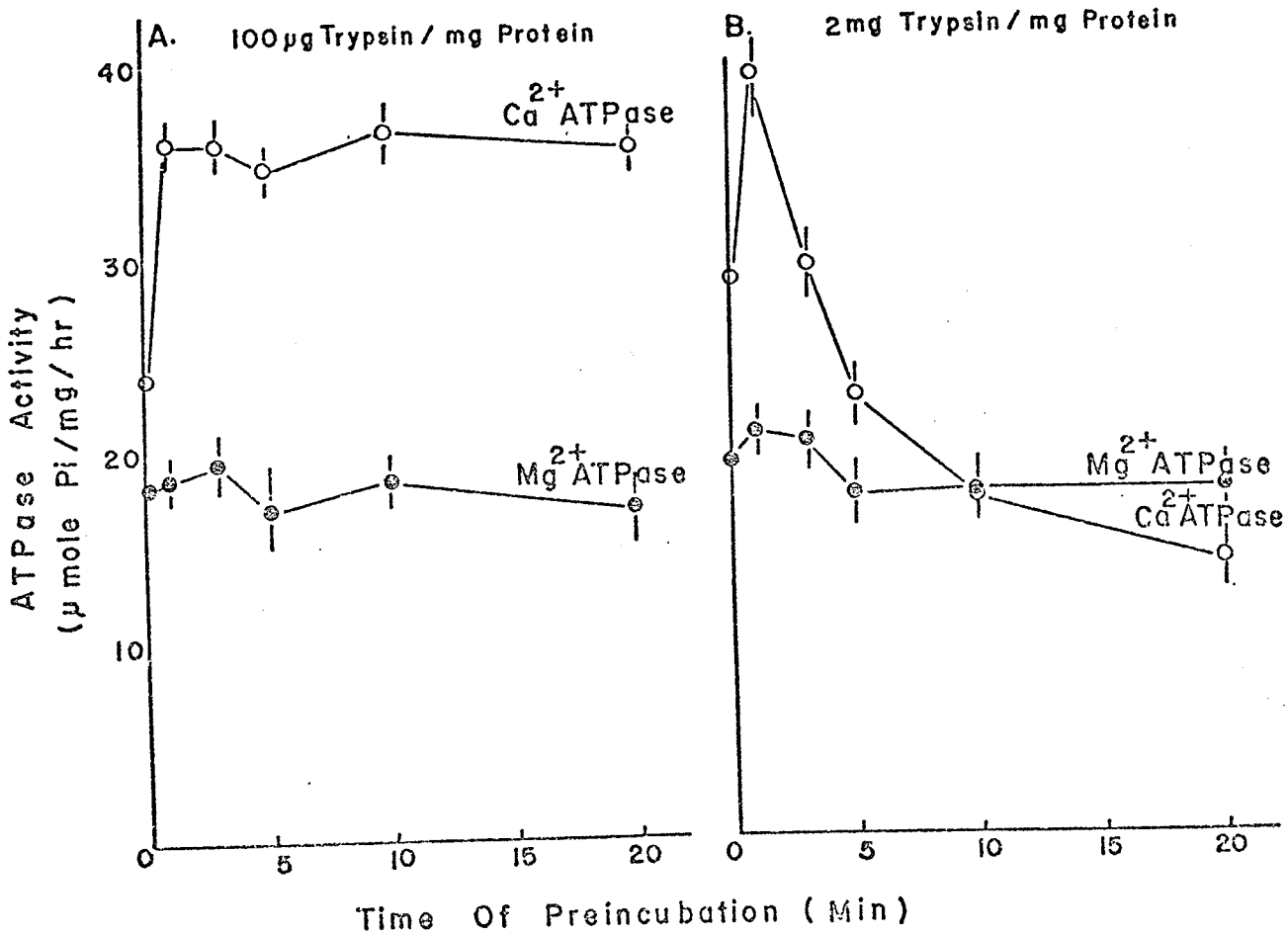

FIGURE 20 Effect of time of preincubation of heart sarcolemma with trypsin on ATP hydrolysis in the presence of 0.25 mM Ca^{2+} or Mg^{2+}. The experimental conditions were the same as those for Fig. 19. Each value is a mean $\pm$ S.E. of 3 experiments.

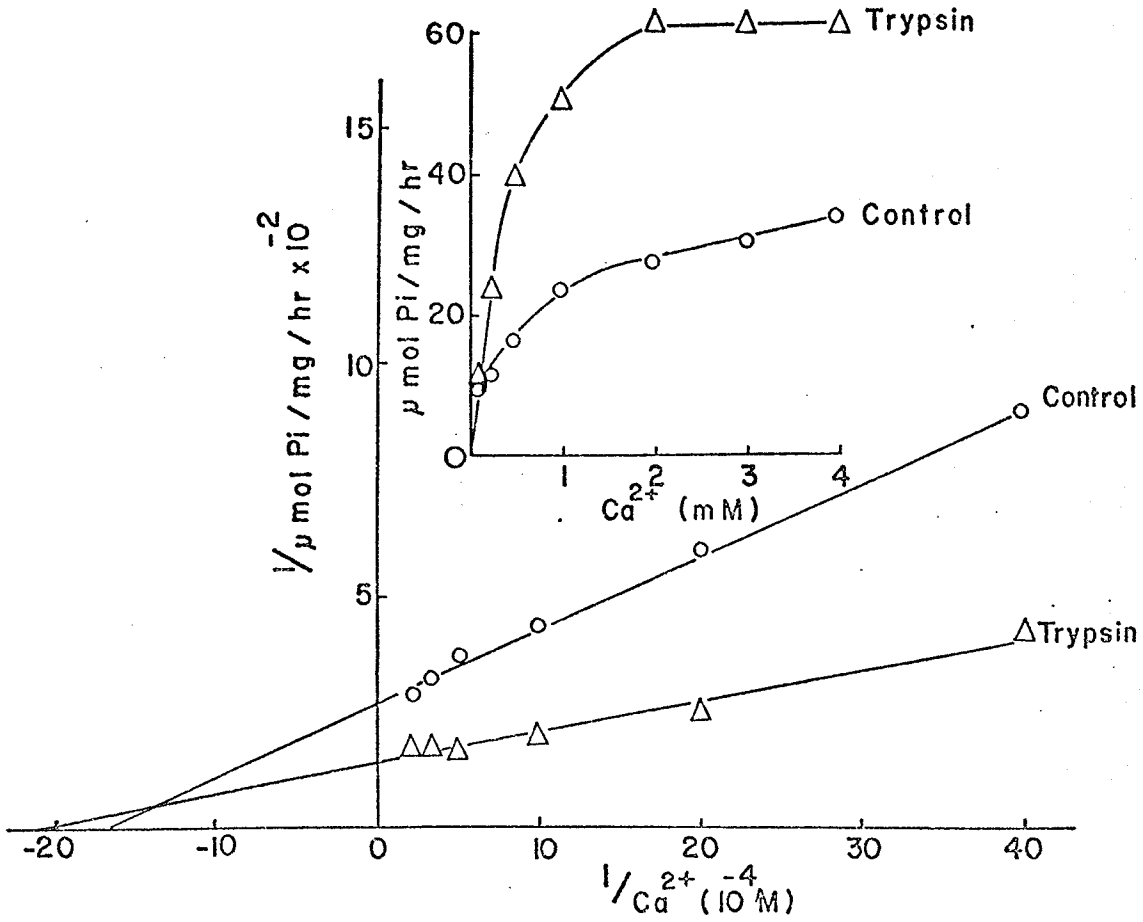

FIGURE 21 Effect of trypsin on ATP hydrolysis by heart sarcolemma in the presence of different concentrations of Ca^{2+}. The experimental conditions were the same as those for Fig. 19 except that ATP concentration was 4 mM. The results are typical of 4 experiments.

TABLE XVI

Effect of Trypsin on Ca^{2+}/Mg^{2+} ATPase Activities of the Detergent Treated Rat Heart Sarcolemma

Amount of trypsin µg/mg sarcolemmal protein	ATPase activities (µmoles Pi released/mg protein/hr)			
	Deoxycholate-treated		Lubrol - treated	
	Ca^{2+} ATPase	Mg^{2+} ATPase	Ca^{2+} ATPase	Mg^{2+} ATPase
Control	30.3 ± 2.1	20.6 ± 1.7	28.8 ± 2.4	13.7 ± 0.9
25	41.8 ± 2.8	18.4 ± 1.5	51.2 ± 3.9	11.9 ± 0.9
50	49.9 ± 3.3	17.4 ± 1.6	54.7 ± 4.1	11.9 ± 0.8
100	53.5 ± 3.2	17.7 ± 1.5	58.1 ± 3.8	11.7 ± 0.7
150	55.6 ± 4.1	16.9 ± 1.2	56.6 ± 3.9	9.6 ± 0.7
200	49.3 ± 3.4	16.5 ± 0.9	51.6 ± 2.7	9.2 ± 0.8
250	45.9 ± 3.2	15.0 ± 0.8	49.9 ± 2.8	9.1 ± 0.5
300	42.0 ± 2.4	14.5 ± 0.8	45.5 ± 2.8	8.8 ± 0.6

The sarcolemma was treated with deoxycholate or lubrol at a concentration of 0.2 µg/mg membrane protein for 10 min at a room temperature, centrifuged and washed thoroughly. This preparation was employed for studying ATPase activities in the absence or presence of different concentrations of trypsin. The concentration of Ca^{2+}/Mg^{2+} or ATP was 1.25 mM. Each value is a mean ± S.E. of 4 different preparations.

a known amount of heart sarcolemma with trypsin. The results in Table XVII indicate about a 2 fold increase in total and specific activities of Ca^{2+} ATPase without any changes in the Mg^{2+} ATPase activities in the presence of trypsin. It should also be noted that the protein content in trypsin-treated membrane preparation was markedly decreased whereas the specific activities for both Ca^{2+} ATPase and Mg^{2+} ATPase activities were increased by 2.5 to 3 fold. The protein released in the supernatant exhibited Ca^{2+} ATPase activity whereas the Mg^{2+} ATPase activity in the supernatant was relatively negligible. The ATP hydrolysis by the supernatant was observed at various concentrations of Ca^{2+}, unlike Mg^{2+}. Lineweaver-Burke analysis of the data (Fig. 22) for the Ca^{2+} ATPase activity of the control heart sarcolemma and the supernatant preparation (obtained after trypsin treatment) revealed K_a values of 0.58 and 0.35 mM and V_{max} values of 37 and 54 µmoles Pi/mg membrane protein/hr respectively. On the other hand, the kinetic data (Fig. 23) for the Ca^{2+} ATPase activity in the trypsin-treated membrane preparation indicated that the V_{max} value increased from 37 to 63 µmoles Pi/mg membrane protein/hr without any changes in the K_a value (0.58 mM). Likewise, an increase in the specific activity of Mg^{2+} ATPase in the trypsin-treated preparation was also associated with an increase in the V_{max} value without any changes in the K_m value (Fig. 24).

For the purpose of this study it was decided to characterize the Ca^{2+}/Mg^{2+} ATPase activities of the trypsin-treated membranes only. For obtaining these preparations with maximal specific enzyme activity, the conditions for trypsin-treated were thoroughly worked out. The results shown in Table XVIII indicate that the presence of different ligands such as Ca^{2+}, Mg^{2+} or ATP at high concentrations in the medium during

TABLE XVII

Influence of Trypsin Digestion on the Heart Sarcolemmal Ca^{2+}/Mg^{2+} ATPase Activities

		Ca^{2+} ATPase		Mg^{2+} ATPase	
	Sarcolemmal protein (mg)	Specific Activity (µmoles Pi/mg/hr)	Total Activity (µmoles Pi/hr)	Specific Activity (µmoles Pi/mg/hr)	Total Activity (µmoles Pi/hr)
Control	5.2	26.0	135.2	20.8	108.2
Trypsin digestion	5.2	50.0	260.0	21.3	110.8
Trypsin-treated membrane	1.8	67.0	120.6	58.2	104.8
Trypsin-treated supernatant	3.2	48.5	155.2	2.5	8.0

Sarcolemma was digested with trypsin at a concentration of 100 µg/mg of protein for 10 min in a medium as given in "Methods." The reaction was stopped by addition of 3 fold trypsin inhibitor. Aliquot of this mixture was taken for ATPase determination (trypsin digestion). The trypsin digested preparation was centrifuged at 1000 x g. The supernatant and residue (after washing twice) were used for ATPase determination. The values for ATPase activities were calculated on the basis of the sarcolemmal protein.

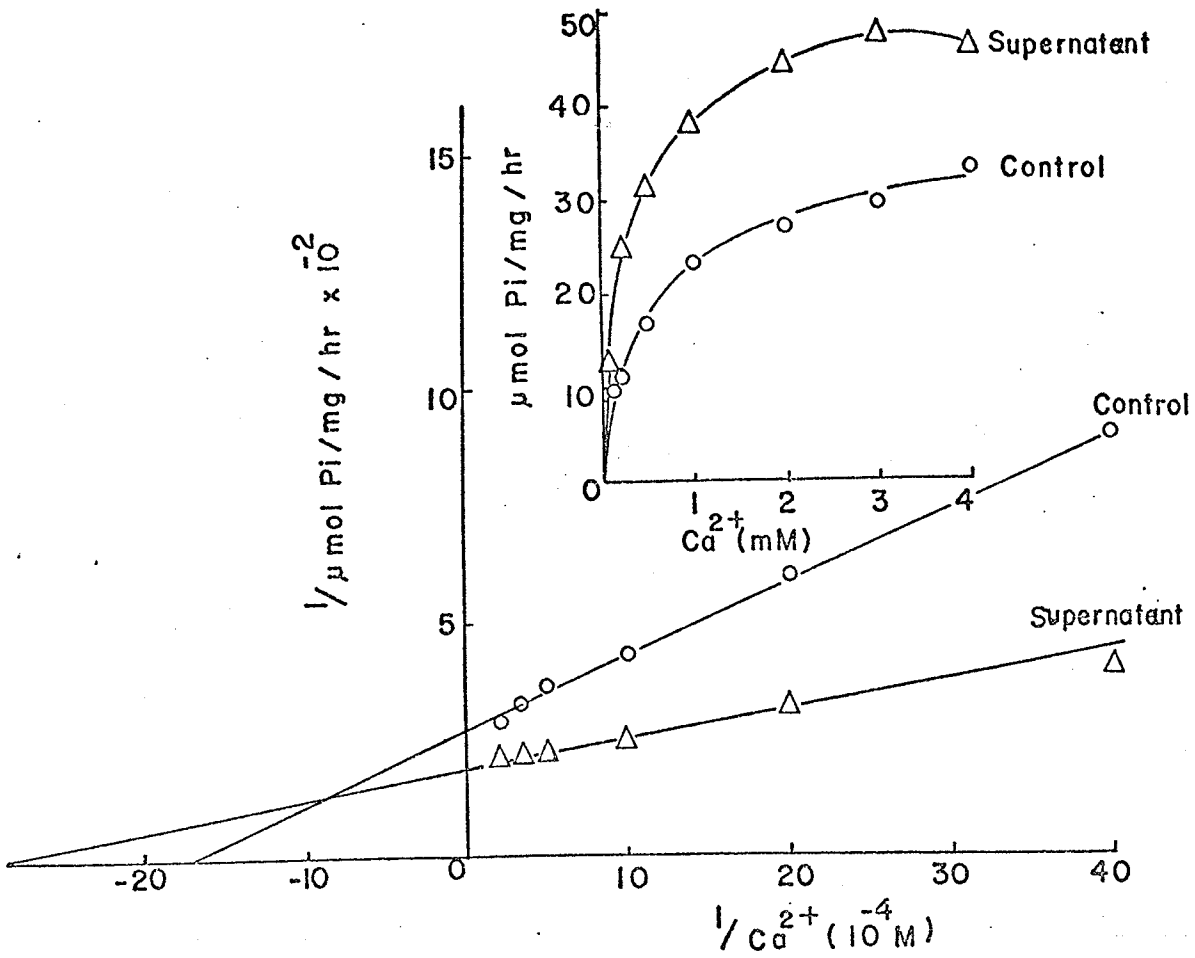

FIGURE 22 Effect of different concentrations of Ca^{2+} on the ATP hydrolyzing activity of the sarcolemmal enzyme released in the supernatant (1000 x g) after trypsin treatment of the sarcolemma. The experimental conditions were the same as those for Fig. 19 except that ATP concentration was 4 mM. Trypsin concentration used for treatment was 100 μg/mg membrane protein. The results are typical of 4 experiments.

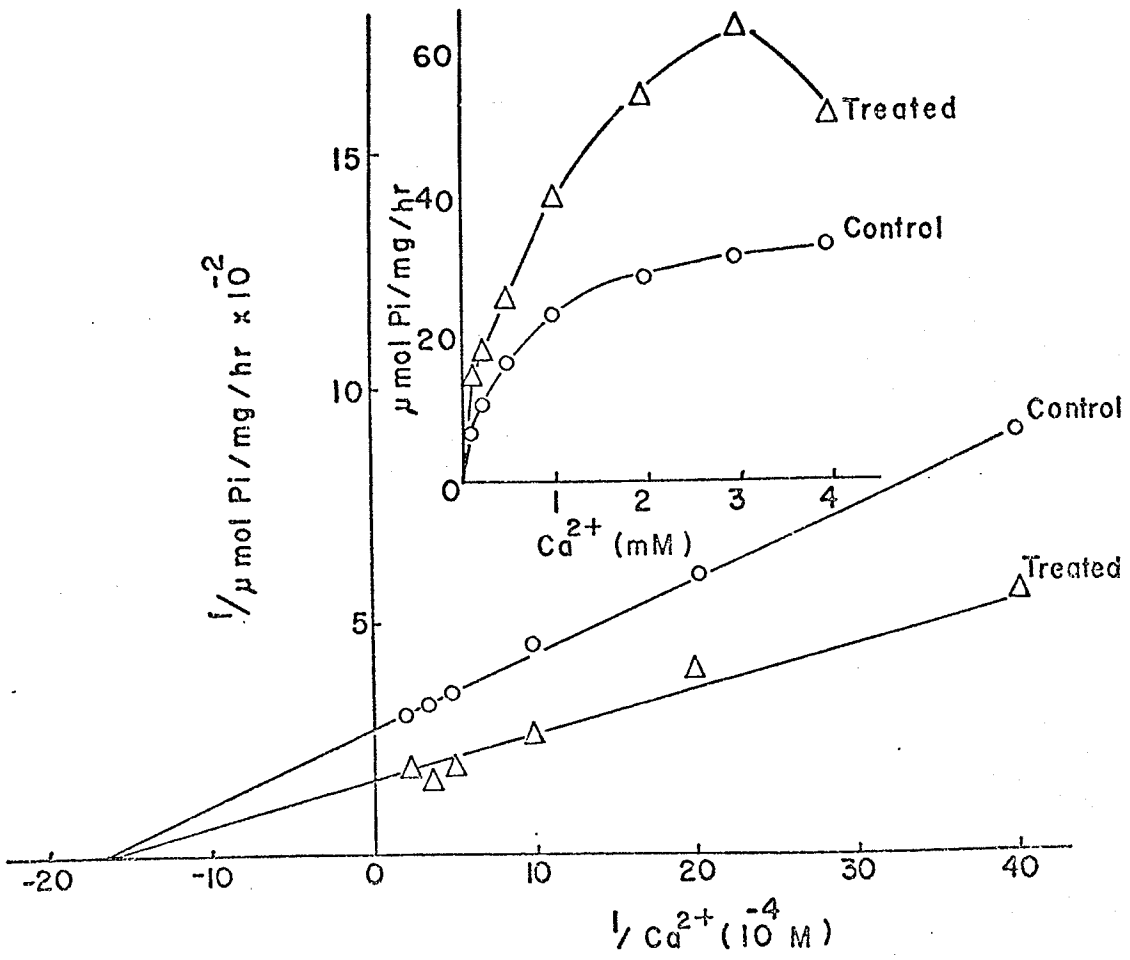

FIGURE 23 Effect of different concentration of Ca^{2+} on ATP hydrolysis by heart sarcolemma pretreated with trypsin at a concentration of 100 μg/mg of protein for 10 min. The experimental conditions were the same as those for Fig. 19 except that ATP concentration was 4 mM. The results are typical of 4 experiments.

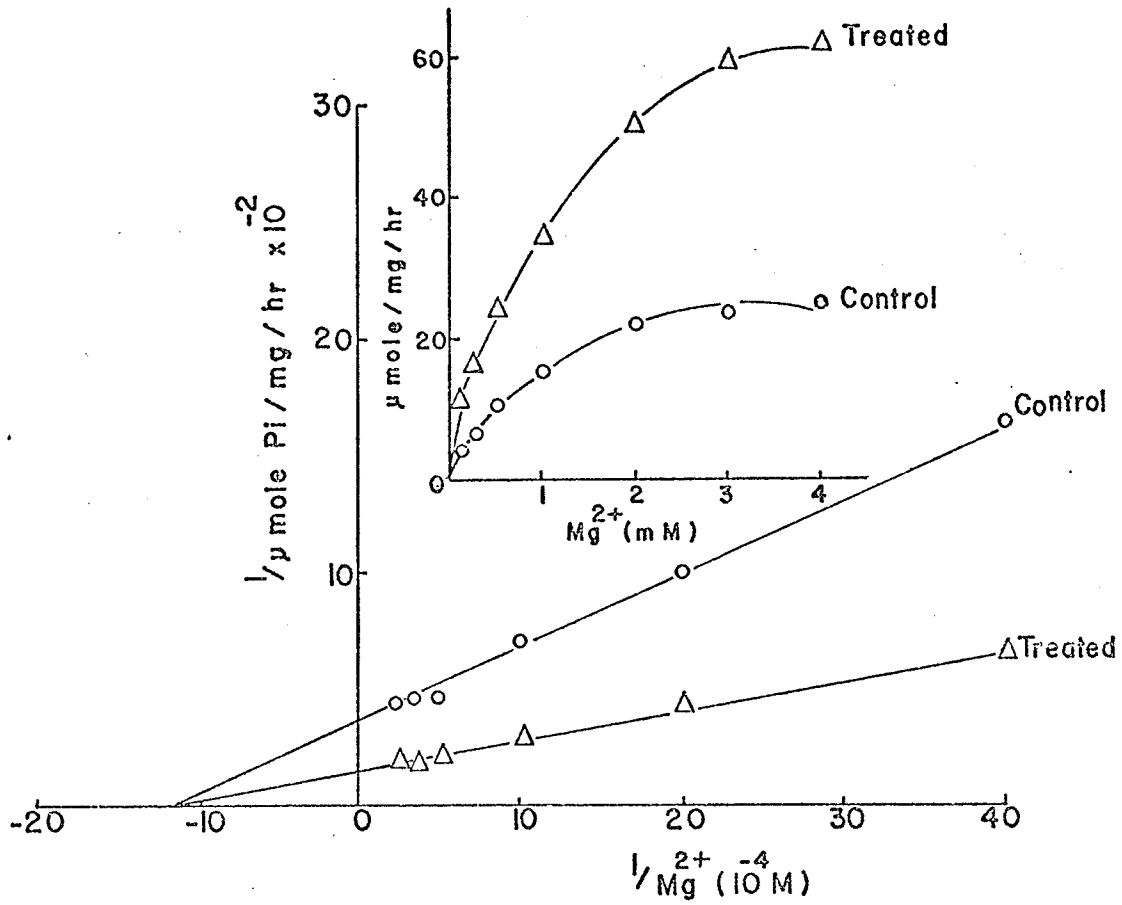

FIGURE 24 Effect of different concentrations of Mg^{2+} on ATP hydrolysis by heart sarcolemma pretreated with trypsin at a concentration of 100 μg/mg of protein for 10 min. The experimental conditions were the same as those for Fig. 19 except that ATP concentration was 4 mM. The results are typical of 4 experiments.

TABLE XVIII

Effect of Trypsin Digestion in the Presence or Absence of Various Ligands on Ca^{2+}/Mg^{2+} ATPase Activities of Heart Sarcolemma

	ATPase activities (μmole Pi released/mg protein/hr)			
	Control		Treated	
Additions	1.25 mM Ca^{2+}	1.25 mM Mg^{2+}	1.25 mM Ca^{2+}	1.25 mM Mg^{2+}
None	30.1 ± 2.2	23.9 ± 1.7	63.9 ± 2.8	63.5 ± 2.7
1 mM Ca^{2+}	32.8 ± 2.1	24.0 ± 1.5	56.8 ± 2.9	55.6 ± 3.2
4 mM Ca^{2+}	32.1 ± 1.8	25.1 ± 1.3	51.3 ± 2.2*	49.9 ± 2.1*
1 mM Mg^{2+}	32.5 ± 1.8	25.0 ± 1.5	57.0 ± 3.0	58.8 ± 1.8
4 mM Mg^{2+}	28.0 ± 1.9	23.2 ± 1.2	52.0 ± 2.4*	50.8 ± 1.8*
1 mM ATP	30.5 ± 2.1	24.0 ± 1.4	59.1 ± 3.1	63.8 ± 2.8
4 mM ATP	30.7 ± 2.3	23.6 ± 1.4	51.2 ± 2.3*	51.3 ± 2.1*

* Significantly different from values obtained in the absence of ligands ($P < 0.05$). Sarcolemma was treated with trypsin at a concentration of 100 μg/mg of membrane protein for 10 min in the presence or absence of divalent cations or ATP. The concentration of ATP for ATPase determination was 1.25 mM. Each value is a mean ± S.E. of 4 experiments.

incubation of heart sarcolemma with trypsin increased the Ca^{2+}/Mg^{2+} activities to a lesser extent in comparison to that seen in their absence. By changing different experimental conditions including changes in pH and cation concentrations of the incubation medium, it was found that the Ca^{2+} ATPase and Mg^{2+} ATPase activities of the trypsin-treated membrane were maximal when the trypsin treatment was carried out in a medium containing 50 mM Tris-HCl, pH 7.5, and 20 mM KCl.

The Ca^{2+}/Mg^{2+} ATPase activities were also measured after digestion of heart sarcolemma with different concentrations of trypsin for 10 min or for different intervals of time by employing 100 µg trypsin/mg membrane protein. The data in Fig. 25 shows that the protein content of the membrane decreased by about 60% after 100 µg trypsin/mg membrane protein and further increase in trypsin concentration did not produce a further decrease in protein content. On the other hand, maximal specific activity of the Ca^{2+} ATPase was achieved at 100 - 150 µg trypsin/mg membrane protein whereas that for the Mg^{2+} ATPase was seen at about 200 µg trypsin/mg membrane protein concentration. Varying the time of digestion indicated that the maximum decrease in membrane proteins was obtained in 10 min whereas the maximal Ca^{2+} ATPase and Mg^{2+} ATPase activities were apparent at 10 and 40 min of digestion with trypsin (Fig. 26).

In another series of experiments, the Ca^{2+}/Mg^{2+} ATPase activities of the trypsin-treated preparations were measured by using different concentrations of Ca^{2+}, Mg^{2+} or ATP. When micromolar concentrations of Ca^{2+} or Mg^{2+} were employed, increases in Ca^{2+} ATPase or Mg^{2+} ATPase activities of the trypsin treated sarcolemma were evident at each concentration (Fig. 27). It was interesting to note that the activation

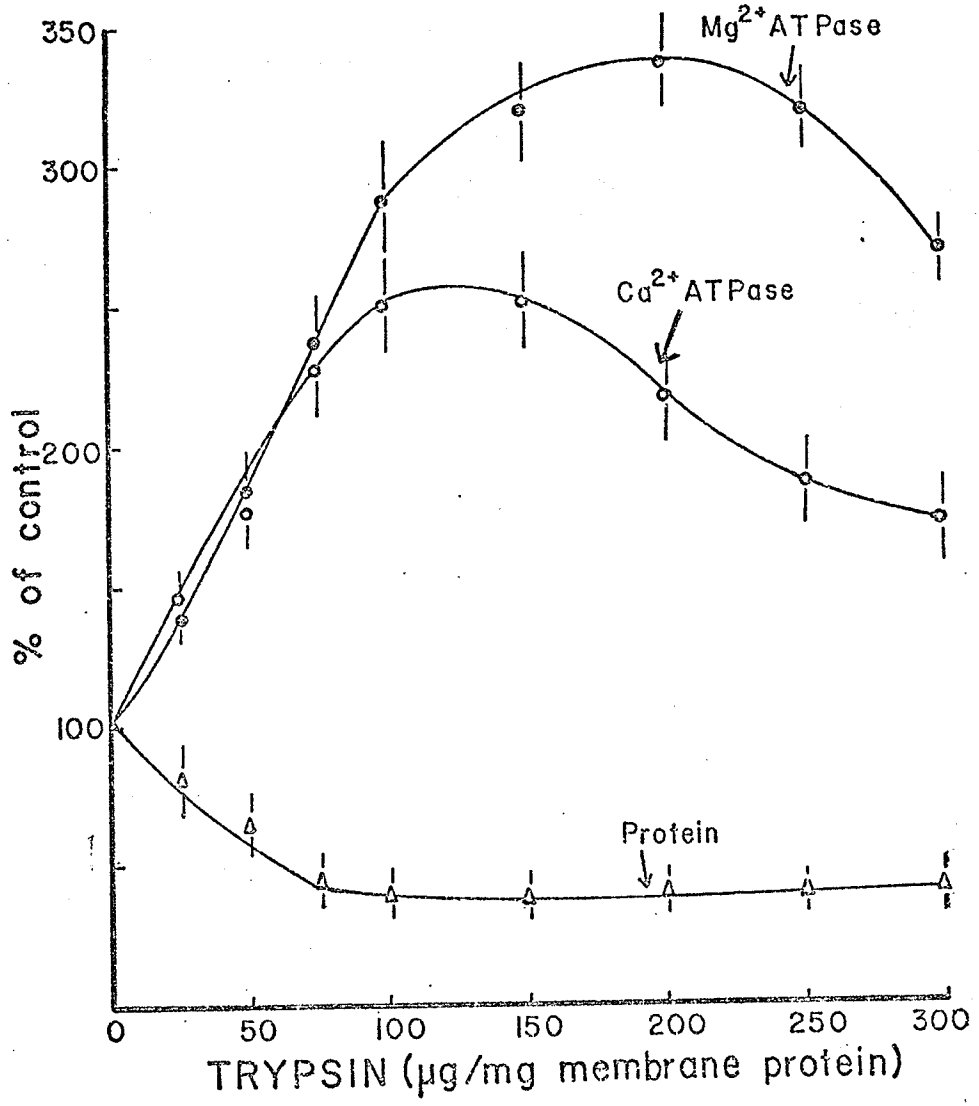

FIGURE 25 Effect of different concentrations of trypsin treatment on Ca^{2+} ATPase, Mg^{2+} ATPase activitis and protein content of heart sarcolemma. The time of incubation with trypsin was 10 min. The concentrations of Ca^{2+} or Mg^{2+} and ATP were 1.25 mM. Each value is a mean $\pm$ S.E. of 4 experiments.

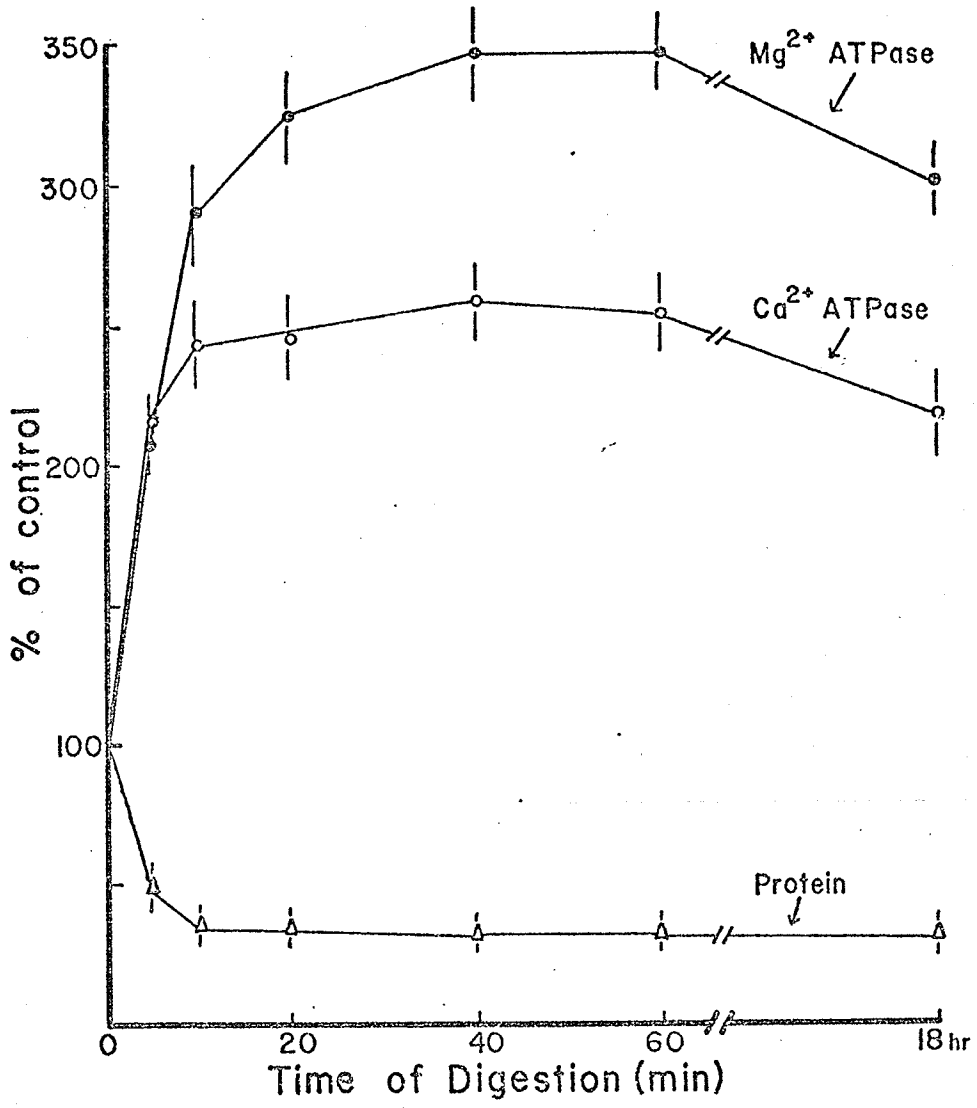

FIGURE 26 Effect of time of digestion of sarcolemma with trypsin at a concentration of 100 µg/mg membrane protein on Ca^{2+} ATPase, Mg^{2+} ATPase activity and protein content. The concentrations of Ca^{2+} or Mg^{2+} and ATP were 1.25 mM. Each value is a mean ± S.E. of 4 experiments.

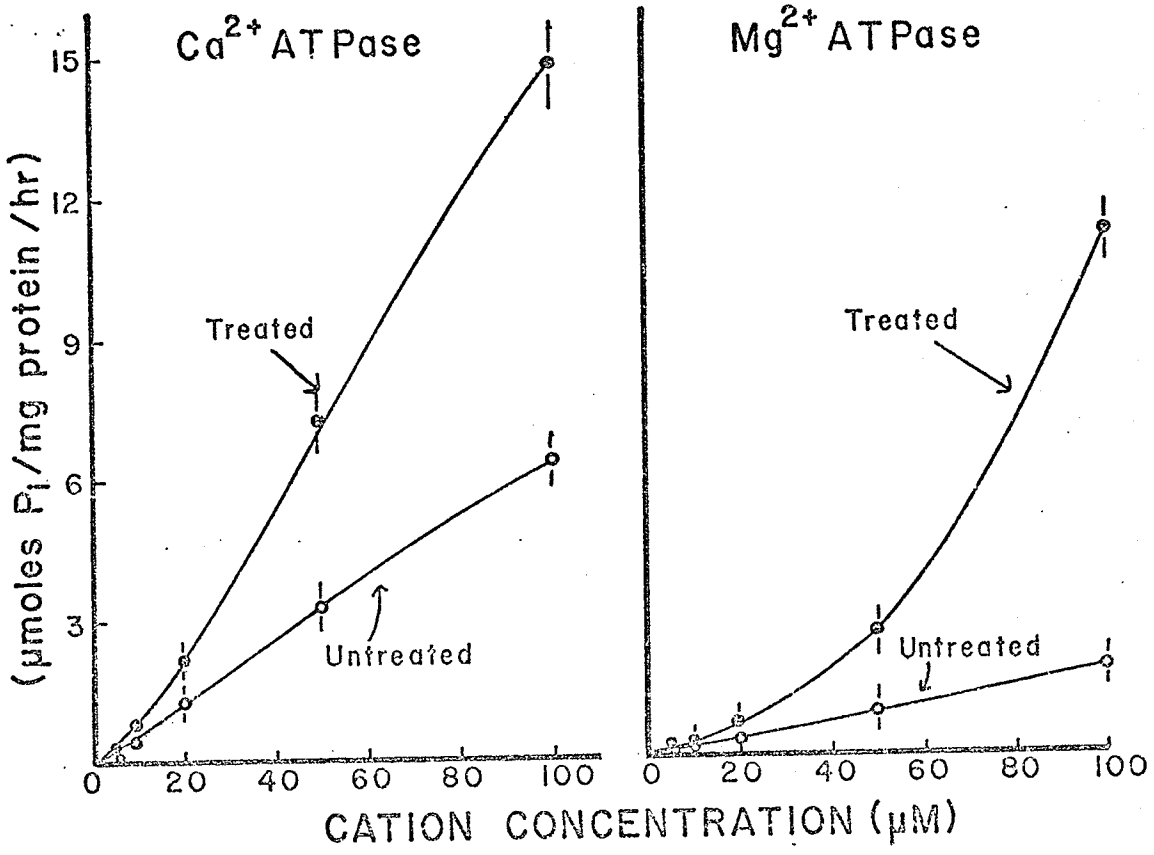

FIGURE 27 Effect of low (μM) concentrations of Ca^{2+} or Mg^{2+} on ATP hydrolysis by heart sarcolemma pre-treated with trypsin at a concentration of 100 μg/mg of protein for 10 min. ATP concentration was 4 mM. The results are the mean ± S.E. of 4 experiments.

pattern of the Mg^{2+} ATPase, unlike that of the Ca^{2+} ATPase, in the trypsin treated preparation gave the appearance like that of an allosteric enzyme. However, no further effort was made to characterize this enzyme system for the present study. The data shown in Fig. 28 indicate that K_m values for the high affinity sites of Ca^{2+} ATPase (68 μM) and Mg^{2+} ATPase (86 μM) of the trypsin-treated membranes were not different from the control. On the other hand, V_{max} values for the high affinity sites of Ca^{2+} ATPase increased from 13 to 50 μmoles Pi/mg/hr and those for Mg^{2+} ATPase increased from 16 to 50 μmoles Pi/mg/hr upon trypsin treatment. ATP hydrolysis was also carried out by employing millimolar concentrations of ATP and the results are shown in Fig. 29. In this case, the K_m value (333 μM) for the low affinity sites for Ca^{2+} ATPase or Mg^{2+} ATPase in trypsin-treated preparation was not different from the control whereas the V_{max} value for Ca^{2+} ATPase increased from 43 to 99 μmoles Pi/mg/hr and that for Mg^{2+} ATPase increased from 30 to 80 μmoles Pi/mg/hr.

The interaction of Ca^{2+} and Mg^{2+} was examined in control and trypsin treated preparations to determine if any changes are produced by one cation when the enzyme is fully activated by the other cation. For this experiment, either Ca^{2+} (4 mM) concentration was remained constant and an increasing amount of Mg^{2+} was added or Mg^{2+} (4 mM) concentration was kept constant and an increasing amount of Ca^{2+} was added. The results shown in Table XIX indicate that Ca^{2+} ATPase activity of the trypsin-treated preparation decreased to a lesser extent by the addition of Mg^{2+} in comparison to the control preparation whereas an equal degree of depression was noted in control and trypsin-treated membrane Mg^{2+} ATPase upon the addition of Ca^{2+}. It should be noted

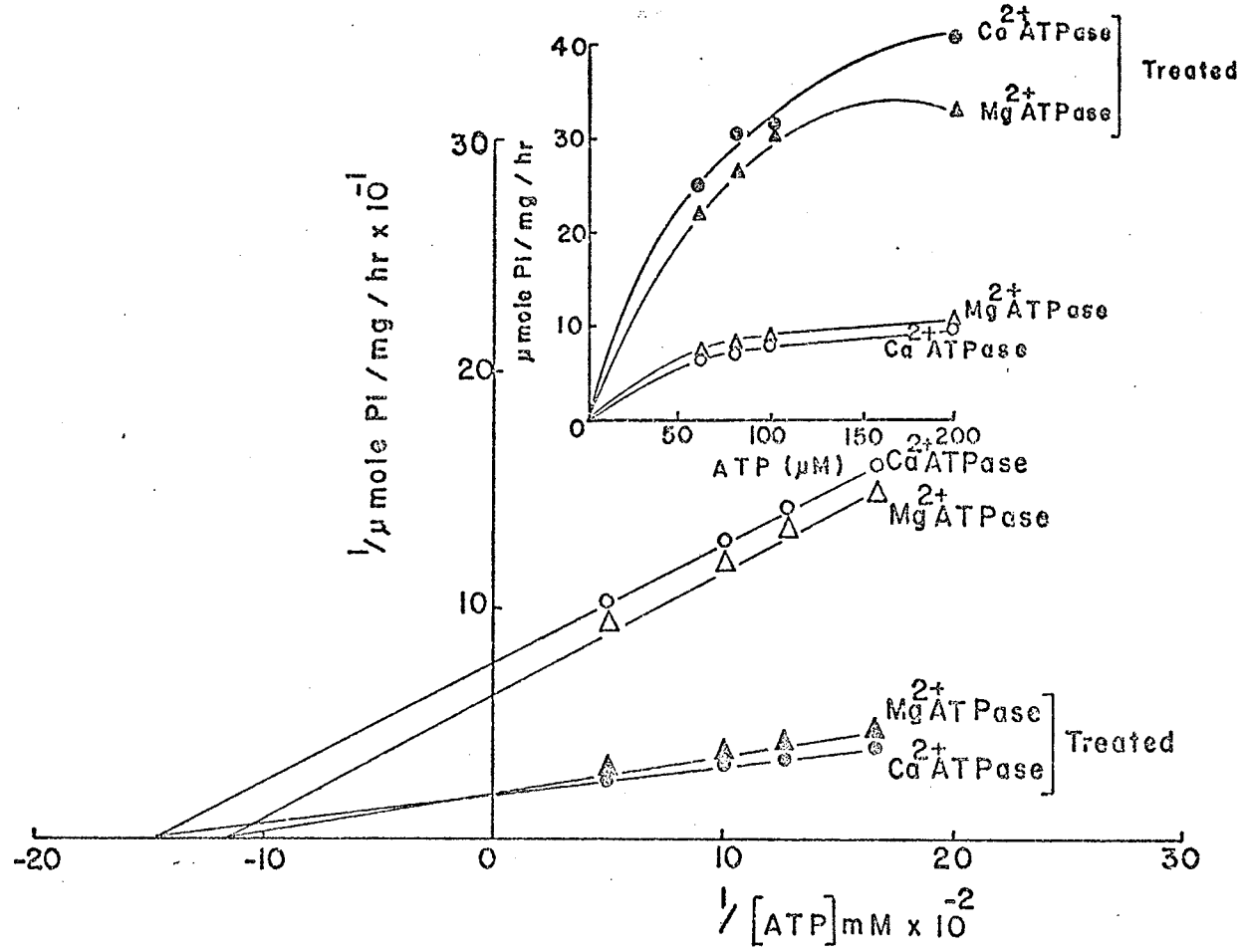

FIGURE 28 Effect of different concentrations of ATP (µM) on ATP hydrolysis by control and trypsin-treated (at a concentration of 100 µg/mg membrane protein for 10 min) heart sarcolemma in the presence of 4 mM Ca^{2+} or Mg^{2+}. The results are typical of 4 experiments.

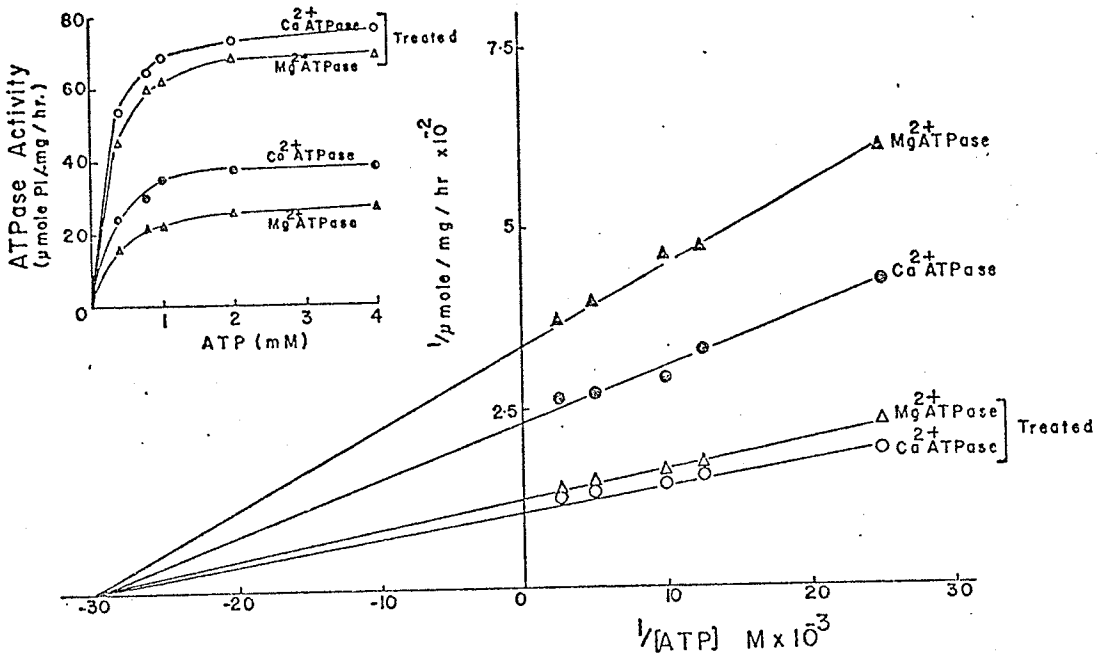

FIGURE 29. Effect of low (m M) concentrations of ATP on ATP hydrolysis by control and trypsin-treated (at a concentration of 100 μg/mg membrane protein for 10 min) heart sarcolemma in the presence of 4 mM Ca^{2+} or Mg^{2+}. The results are typical of 4 experiments.

TABLE XIX.

Effect of Ca^{2+} or Mg^{2+} on Ca^{2+}/Mg^{2+} ATPase Activities from Control and Trypsin Treated Sarcolemma

	ATPase activity (% of control)			
	Ca^{2+} ATPase		Mg^{2+} ATPase	
Additions Ca^{2+} or Mg^{2+}	Control	Treated	Control	Treated
50 µM	86.5 ± 6.2	104.4 ± 5.9	100.7 ± 3.7	98.3 ± 3.2
100 µM	77.2 ± 6.7	101.5 ± 4.7	96.4 ± 2.9	94.3 ± 3.4
0.5 mM	62.8 ± 8.9	104.1 ± 4.8	93.5 ± 2.8	96.5 ± 3.5
1.0 mM	63.7 ± 8.7	98.7 ± 3.2	91.5 ± 2.8	92.7 ± 2.6
2.0 mM	61.4 ± 7.5	95.2 ± 5.1	88.8 ± 3.0	88.9 ± 2.8
3.0 mM	59.2 ± 6.2	93.3 ± 5.6	85.9 ± 3.1	81.1 ± 2.5
4.0 mM	57.3 ± 6.0	87.4 ± 6.9	81.9 ± 3.6	77.5 ± 2.6

ATP concentration in the incubation medium was 4 mM. Ca^{2+} ATPase and Mg^{2+} ATPase activities were monitored in the presence of 4 mM Ca^{2+} or Mg^{2+}. Different concentrations of Mg^{2+} were added to the assay medium for Ca^{2+} ATPase activity whereas different concentrations of Ca^{2+} were added to the assay medium for Mg^{2+} ATPase activity. Each value is a mean ± S.E. of 4 experiments.

that the pH optima for the Ca^{2+} ATPase or Mg^{2+} ATPase activities in the trypsin-treated preparations were similar to those of the control membranes (Fig. 30). Furthermore, 5 to 100 mM concentrations of Na^{+} or K^{+} did not significantly affect the Ca^{2+} ATPase or Mg^{2+} ATPase activities in the control and trypsin-treated preparations (data not shown).

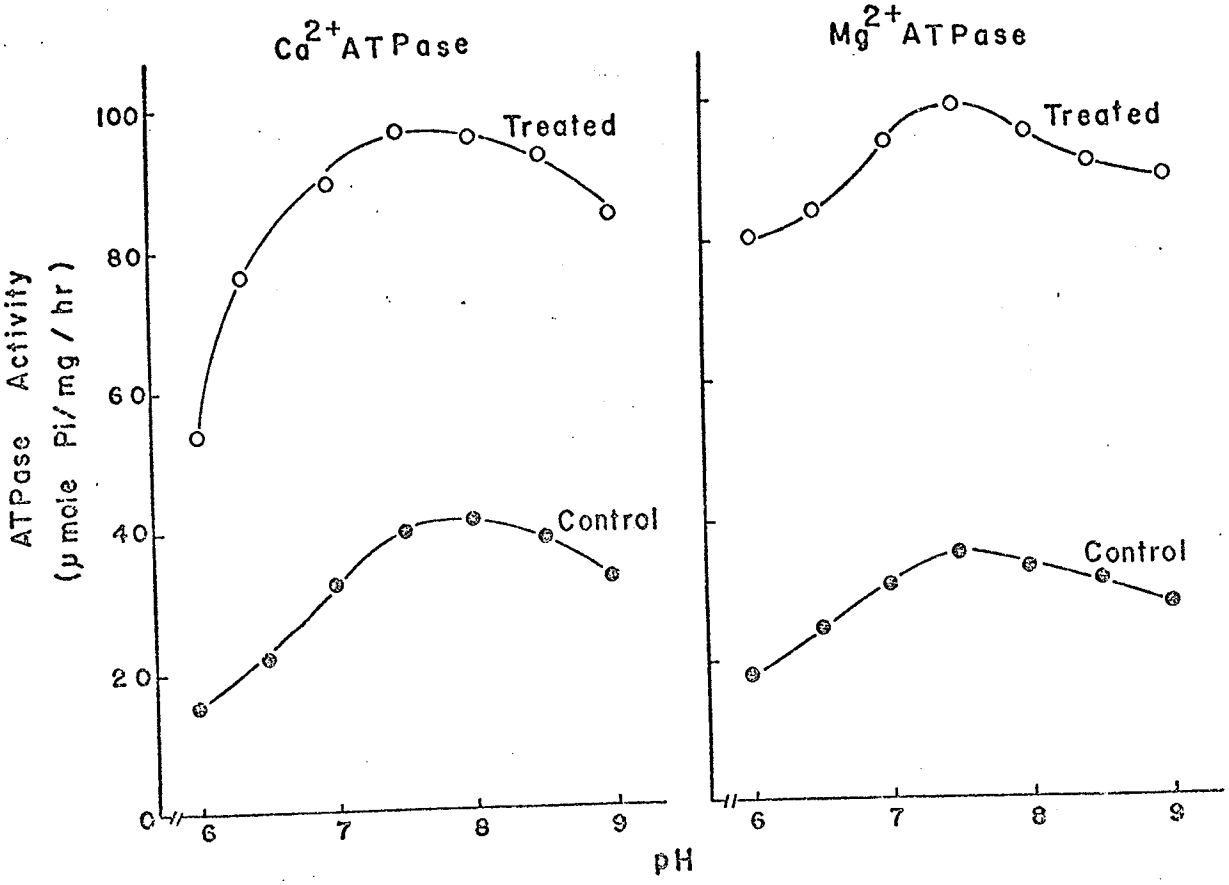

FIGURE 30 pH profiles of the ATP hydrolyzing activities of control and trypsin-treated heart sarcolemma in the presence of 4 mM Ca^{2+} or Mg^{2+}. Trypsin treatment of sarcolemma was at a concentration of 100 μg/mg membrane protein for 10 min. The concentration of ATP was 4 mM. Each value is an average of 3 experiments.

V. DISCUSSION

A. Heart Sarcolemmal, Mitochondrial and Microsomal Ca^{2+}/Mg^{2+} ATPase:

In this study we have demonstrated that various divalent cations stimulate ATP hydrolysis by sarcolemma, mitochondria and microsomes, however the order of their potency for these membrane fractions was different from each other. Although concentrations of Ca^{2+} or Mg^{2+} required for maximal ATPase activities of heart sarcolemma were similar to those for the mitochondrial and microsomal fractions, pH optimum for the sarcolemmal ATPase activity varied between 7.5 to 8.0 whereas that for mitochondrial or microsomal enzyme activity was between 8.0 to 8.5. These results indicate some differences between ATPase systems of mitochondria, microsomes and sarcolemma. This view is substantiated by the fact that mitochondrial ATPases, unlike those of microsomes and sarcolemma, were stimulated and depressed by DNP and sodium azide respectively. Furthermore, ruthenium red was most effective in decreasing mitochondrial ATPase activities and least effective in supressing the sarcolemmal ATPase activities. The degrees of depression in mitochondrial, microsomal and sarcolemmal ATPase activity by NaF were found to be different with each membrane. The activities of mitochondrial ATPases decreased to a greater extent in comparison to the microsomal ATPases by PCMB whereas low concentrations of lanthanum inhibited sarcolemmal ATPase activities only. Although there is a good possibility that the observed differences are truely due to the presence of different ATPases in these membranes, the conclusive answer must await a comparative study of these enzyme systems after the methods for their isolation from these membranes and purification are perfected.

In spite of the finding that ATP hydrolysis by different

heart membranes is stimulated by Mg^{2+} or Ca^{2+}, it is not clear whether these cations produce this effect by stimulating the same or different enzyme complex. In this regard it should be noted that the magnitudes of stimulation of ATP hydrolysis by mitochondria and microsomes due to Mg^{2+} were almost identical to those seen with Ca^{2+}; the sarcolemmal Ca^{2+} ATPase activity was observed to be more than the Mg^{2+} ATPase activity. Furthermore, the Ca^{2+} ATPase activities of mitochondrial, microsomal and sarcolemmal fractions were inhibited to a greater extent by agents such as NaF, ruthenium red and iodoacetate in comparison to the Mg^{2+} ATPase activities. The inhibitory effect of sodium azide on mitochondrial Mg^{2+} ATPase was greater than that on the Ca^{2+} ATPase activity. Iodoacetate was capable of stimulating mitochondrial Mg^{2+} ATPase activity only, whereas iodoacetamide and maleic anhydride stimulated the sarcolemmal and mitochondrial Mg^{2+} ATPase activities respectively without affecting the ATP hydrolysis by these membranes in the presence of Ca^{2+}. On the other hand, carbodiimide and maleic anhydride decreased mitochondrial and sarcolemmal Ca^{2+} ATPase activities respectively. Furthermore, microsomal Mg^{2+} ATPase activities but not Ca^{2+} ATPase activities were decreased by iodoacetamide and carbodiimide. A biphasic effect on the sarcolemmal Mg^{2+} ATPase was exerted by PCMB which showed only inhibitory effects on the Ca^{2+} ATPase. These diverse type of responses of the membrane ATPases to agents, which react with sulfhydryl, amino and carboxyl groups, support the view that these ATPases may in certain aspects be different from each other.

From the foregoing discussion it appears that mitochondrial, microsomal and sarcolemmal membranes may contain different ATPases which are activated by various divalent cations and are possibly isoenzymes (224).

Since some of the inhibitors employed in this study altered ATPase activities of heart membranes to varying degrees, it can be argued that such results are due to some cross contamination of the cellular fractions. However, this view is not supported by electron microscopic, marker enzyme, and other physico-chemical studies with these fractions and it may be that such actions are due to the lack of specificity of these agents. At any rate, the interaction of mitochondrial ATPases with DNP and sodium azide as well as of sarcolemmal ATPase with lanthanum suggest that these ATPase systems may play an important role in regulating the functions of these membranes.

B. Comparison of Heart Sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase:

It is clear from this study that the specific activity of the heart sarcolemmal Ca^{2+} ATPase was higher than the Mg^{2+} ATPase activity. This difference in the ability of sarcolemma to hydrolyze ATP in the presence of Ca^{2+} and Mg^{2+} can be conceived to be due to differences in the affinities of cations or ATP for the enzyme complex. In this regard, it should be noted that the K_a value for Ca^{2+} ATPase was found to be lower than that for Mg^{2+} ATPase. Although K_m values for the low affinity sites were the same for both ATPases, the K_m value for the high affinity sites for Ca^{2+} ATPase was lower than that for Mg^{2+} ATPase. Furthermore, the V_{max} values for the low affinity sites, but not for the high affinity sites, for Ca^{2+} ATPase were higher than for Mg^{2+} ATPase. It is pointed out that the observed difference in the behaviour of sarcolemma in the presence of Ca^{2+} and Mg^{2+} was not only limited to ATP hydrolysis because the hydrolysis of other nucleotides such as GTP, UTP, CTP and AMP in the presence of Ca^{2+} was also different from that in the presence of Mg^{2+}. These results can be interpreted to mean that either sites involved in ATP hydrolysis in the presence of Ca^{2+} and Mg^{2+}

are different from each other or two different enzyme systems exhibiting Ca^{2+} ATPase and Mg^{2+} ATPase activities are present in heart sarcolemma. These questions can only be settled when pure preparations of these enzyme systems will become available.

In this study it was observed that the sarcolemmal Ca^{2+} ATPase activity was inhibited to a lesser extent by ADP and to a greater extent by Pi in comparison to the Mg^{2+} ATPase activity. Furthermore, the increase in Ca^{2+} ATPase activity was greater than that of Mg^{2+} ATPase activity upon storing heart sarcolemma at 0 - 2°C for different intervals. On the other hand, both freezing and thawing as well as preheating the sarcolemmal preparation decreased the Ca^{2+} ATPase and Mg^{2+} ATPase activities to an equal extent. In addition, neither Ca^{2+} ATPase nor Mg^{2+} ATPase activity was affected by the presence of different monovalent cations. Although the degrees of inhibition of Ca^{2+} ATPase and Mg^{2+} ATPase activities by divalent cations such as Cu^{2+}, Ni^{2+} and Co^{2+} were similar for these enzyme systems, it should be noted that the inhibitory effect of Mn^{2+} on Ca^{2+} ATPase was greater than that on the Mg^{2+} ATPase. These results indicate some differences and similarities in the properties of the heart sarcolemmal Ca^{2+} ATPase and Mg^{2+} ATPase.

Since divalent cations such as Ni^{2+}, Mn^{2+} and Co^{2+} are known to depress myocardial contractility by inhibiting calcium currents (19, 20), the observed decrease in Ca^{2+} ATPase activity by these cations can be interpreted to indicate that Ca^{2+} ATPase may be involved in calcium influx in some unknown manner. In this regard, it should be pointed out that the sarcolemmal Ca^{2+} ATPase has to be implicated in opening the calcium channels upon excitation of the myocardial cell (136). Furthermore, verapamil, which is known to inhibit calcium currents in depolarized

myocardium, failed to affect the sarcolemmal Ca^{2+} ATPase under basal conditions. On the other hand, Ca^{2+} ATPase activity was increased by phosphorylating heart sarcolemma by treatments with cyclic AMP-protein kinase. This observation appears to have an important bearing on the mechanisms responsible for the positive inotropic agents, such as catecholamines, which are known to increase cyclic AMP in the myocardium. It is now well known that catecholamines increase calcium influx through the involvement of cyclic AMP-protein kinase mediated phosphorylation (136) and it is possible that this event is associated with an increase in the sarcolemmal Ca^{2+} ATPase activity. Although changes exerted by cyclic AMP - protein kinase as well as divalent cations were not specific for the sarcolemmal Ca^{2+} ATPase because the Mg^{2+} ATPase activity was also affected similarly, further work concerning the involvement of Ca^{2+} ATPase in the process associated with calcium influx is necessary for any meaningful conclusion.

C. Role of Membrane Phospholipids in Ca^{2+}/Mg^{2+} ATPase Activity:

Phospholipases have been widely used in studies concerning the lipid requirement of the (Ca^{2+} - Mg^{2+}) - ATPase in the sarcoplasmic reticulum (166, 179, 183) and erythrocyte (171 - 173) membranes. There is considerable evidence that phospholipases are able to hydrolyze specifically phospholipid substrates which are attached to biological membranes and thus change the enzyme activity. In view of the specificity of their action and the generally mild conditions under which the treatment can be carried out, phospholipases are thus ideal tools for the selective modification of membrane structure by elimination or alteration of a specific group of phospholipids. In this study, we have demonstrated that the treatment of sarcolemma with all the three phospholipases A, C and D caused a decrease in Mg^{2+} ATPase activity. In this regard, phos-

pholipase A from Naja naja, which degrades glycerophospholipids to lyso-derivatives and free fatty acids (179, 183), resulted in about 70% reduction of the Mg^{2+} ATPase activity. On the other hand, the treatment with phospholipase C from C. Welchii, which is specific for lecithin and sphinogymyelin (225, 226) and phospholipase D, which hydrolyzes the phospholipid into phosphatidic acid (170, 183), inhibited Mg^{2+} ATPase activity to about 20% only. It was interesting to note that the Ca^{2+} ATPase activity was not much influenced by phospholipase C and D treatments whereas phospholipase A treatment inhibited Ca^{2+} ATPase activity to about 20%. The reduction in Ca^{2+} ATPase and Mg^{2+} ATPase activity due to phospholipase A treatment thus suggests that glycerophospholipids are required for mainly for the Mg^{2+} ATPase and to some extent for the Ca^{2+} ATPase activities. Although lyso-compounds and free fatty acids produced by phospholipase A treatment have been shown to remain quantitatively in the membrane (174), the reduction in ATPase activity of the phospholipase treated preparation can not be attributed to the fatty acids, because both saturated or unsaturated fatty acids were shown to have no effect on Ca^{2+} ATPase or Mg^{2+} ATPase activities. It should be noted that some investigators have reported the inhibitory effect of free fatty acids on Ca^{2+}/Mg^{2+} ATPase activity in other tissues (100). At any rate, the results concerning the treatment of sarcolemma with phospholipase C and D indicate that glycerophospholipid (lecithin), sphingomyelin and phosphatidic acid are also involved in Mg^{2+} ATPase activity but not in Ca^{2+} ATPase activity.

On the basis of results concerning changes in the ATPase activities of heart sarcolemma upon treatments with phospholipases, it is tempting to postulate that the phospholipids required for the Ca^{2+}

ATPase activity are in some way different than those required for the Mg^{2+} ATPase activity. It is also possible that the phospholipids associated with Ca^{2+} ATPase may not be completely exposed outside and thus are resistant to phospholipase attack. This can further be substantiated by the fact that the Ca^{2+} ATPase activity was decreased by varying degrees upon exposing the leaky membranes (obtained by a mild deoxycholate treatment) to phospholipases. Differences in the accessibility of hydrophilic portion of lecithin associated in sarcoplasmic reticulum membrane to enzymatic attack by phospholipase C and D has also been reported by Martonosi et al. (179). At any rate, the results of this study indicate that phospholipase treatments increased the K_a value for Mg^{2+} but the V_{max} value for phospholipase A treated preparation was decreased, that for phospholipase D preparation was increased and that for phospholipase C preparation did not change. Furthermore, our inability to reactivate Mg^{2+} ATPase after delipidation by phospholipase A treatment by different synthetic phospholipids like lecithin, lysolecithin and phosphatidic acid may be due to an incomplete removal of phospholipase from the membrane after repeated washing (227) or extensive irreversible damage to the phospholipid-enzyme arrangement under the experimental conditions employed in this study.

D. Role of Membrane Protein in Ca^{2+}/Mg^{2+} ATPase Activity:

Trypsin has been shown to increase the heart sarcolemmal Ca^{2+} ATPase activity without appreciably altering the Mg^{2+} ATPase activity. This increase in Ca^{2+} ATPase activity was found to be associated with a decrease in the K_a value and an increase in the V_{max} value. From these data it appears that the orientation of these two enzyme systems in the sarcolemmal membrane may be different from each other. The protein associated with the Ca^{2+} ATPase activity may be located at the outer

surface of the membrane and this peripheral protein is thus easily accessible to trypsin. On the other hand, the protein associated with the Mg^{2+} ATPase activity may be located at the inner surface of the membrane and this integral protein is thus resistant to the proteolytic attack. This view is supported by our finding that the Mg^{2+} ATPase activity, unlike the Ca^{2+} ATPase activity, was decreased by trypsin when deoxycholate or lubrol treated preparations were employed. Furthermore the trypsin induced stimulation of the Ca^{2+} ATPase activity reached to a maximal level within one minute whereas the Mg^{2+} ATPase activity was not appreciably affected even after a prolonged digestion with trypsin. In addition, the Ca^{2+} ATPase, unlike the Mg^{2+} ATPase, was released into the supernatant upon treating the heart sarcolemma with trypsin.

The experiments described in this study reveal that the specific activities of the Ca^{2+} and Mg^{2+} ATPases in trypsin-treated preparations were increased by 2.5 and 3 fold. This change was associated with alterations in morphological appearance as well as electrophoretic pattern of heart sarcolemma and can be explained mainly on the basis of a decrease in membrane proteins. However, it should be noted that the maximal Ca^{2+} ATPase and Mg^{2+} ATPase activities were seen when 100 - 150 µg and about 200 µg/mg membrane protein concentrations of trypsin were employed respectively whereas tryspin concentrations greater than 100 µg/mg membrane protein did not produce any further loss of membrane proteins. Likewise, the maximal Ca^{2+} ATPase and Mg^{2+} ATPase activities were observed in 10 and 40 min of incubating heart sarcolemma with 100 µg trypsin/mg protein respectively. Thus trypsin can be seen to produce some different types of direct effects on the Ca^{2+} ATPase and Mg^{2+} ATPase systems of heart sarcolemma. In this context it should be noted that trypsin has been reported to inhibit the sarcoplasmic reticular Ca^{2+} stimulated

ATPase activity by cleaving the ATPase protein itself (204). Alternatively, there appears to be some degree of difference for Ca^{2+} ATPase and Mg^{2+} ATPase activities with respect to the protein-protein interaction in the heart sarcolemmal membrane.

The observed increase in the specific activities of Ca^{2+} ATPase and Mg^{2+} ATPase in trypsin-treated sarcolemma was found to be associated with an increase in their V_{max} values without any changes in their K_a value. Likewise, no changes in the K_m values for both the low and high affinity sites were apparent whereas their V_{max} values were increased in trypsin-treated preparations. Furthermore, these changes in the enzyme activities in trypsin-treated preparations were not due to any alterations in their pH optima. The inhibitory response of Ca^{2+} ATPase in trypsin-treated preparations to Mg^{2+} was also similar to that of Mg^{2+} ATPase to Ca^{2+}. In spite of these similarities between Ca^{2+} ATPase and Mg^{2+} ATPase systems of trypsin-treated heart sarcolemma, some differences in these enzyme were evident. For example, the K_m value for the high affinity sites of Ca^{2+} ATPase was lower than that for the Mg^{2+} ATPase. The activation pattern of the Mg^{2+} ATPase in trypsin-treated preparations at low concentrations of Mg^{2+}, unlike that for the Ca^{2+} ATPase, was readily identifiable for its allosteric nature since the curve was sigmoidal in shape. These rather minor differences in results can, however, be explained on the basis of some differences in the accessibility of Ca ATP and Mg ATP for the active enzyme sites in the membrane preparation. Alternatively, some trypsin-sensitive Ca^{2+} ATPase, which was released in the supernatant during trypsin digestion, may still be present in the trypsin-treated preparations employed in this study. At any rate, the results presented here provide some evidence that two types of divalent cation stimulated ATPases, namely trypsin-

sensitive and trypsin-insensitive ATPases, are present in the heart sarcolemma. The trypsin-sensitive ATPase is stimulated by Ca^{2+} only whereas the trypsin-insensitive ATPase is stimulated by either Ca^{2+} or Mg^{2+}. Further studies concerning the isolation, purification and characterization of these two ATPase systems will hopefully extend our knowledge on heart sarcolemma and its function.

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