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PURIFICATION AND CHARACTERIZATION OF Ca^{2+}/Mg^{2+} ATPase OF RAT HEART PLASMA MEMBRANE

A Thesis

Presented to the

University of Manitoba

In Partial Fulfilment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

DAYUAN ZHAO

Department of Physiology

Faculty of Medicine

June 1989

PURIFICATION AND CHARACTERIZATION OF Ca^{2+}/Mg^{2+} ATPase OF RAT HEART PLASMA MEMBRANE

BY

DAYUAN ZHAO

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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CONTENTS

LIST OF FIGUERS

LIST OF TABLES

Purification and Characterization of Ca^{2+}/Mg^{2+} ATPase from Heart Plasma Membrane

ABSTRACT

The presence of a divalent cation dependent ATPase (Ca^{2+}/Mg^{2+} ATPase) in the cell membrane has been demonstrated previously. In this study the activation of Ca^{2+}/Mg^{2+} ATPase was examined by employing rat heart plasma membranes. The membrane-bound Ca^{2+}/Mg^{2+} ATPase was activated by millimolar concentrations of Ca^{2+} or Mg^{2+}; some other divalent cations also activated the enzyme but to a lesser extent. Different divalent cations, except Ba^{2+}, depressed the enzyme activity in the presence of Ca^{2+} or Mg^{2+}. The rate of ATP hydrolysis by Ca^{2+}/Mg^{2+} ATPase decayed exponentially; the first-order rate constants were 0.14 - 0.18 min^{-1} for Ca^{2+}-ATPase activity and 0.15 - 0.30 min^{-1} for Mg^{2+} ATPase at 37^oC. The inactivation of the enzyme depended upon the presence of ATP or other high energy nucleotides but was not due to the accumulation of products of ATP hydrolysis. Furthermore, the inactivation of the enzyme was independent of temperature below 37^oC. Concanavalin A, which interacts with carbohydrate residues, when added into the incubation medium before ATP blocked the ATP-dependent inactivation; this effect was prevented by α-methylmannoside. These results suggest that the Ca^{2+}/Mg^{2+} ATPase may be regulated by ATP.

The Ca^{2+}/Mg^{2+} ATPase was solubilized from rat heart plasma membrane by employing 5 mg/ml lysophosphatidylcholine, 5

mg/ml CHAPS, 0.6M NaI, 1mM EDTA, 50 mM Tris-HCl, pH 7.4. The enzyme was purified by sucrose density gradient, Affi-Gel Blue column and Sepharose 6B column chromatography and was seen as a single peptide band in the sodium dedocyle sulfate polyacrylamide gel electrophoresis with a molecular weight of about 90,000. The molecular weight of the enzyme as determined by gel filtration on Sepharose 6B column was about 180,000. The purified enzyme hydrolyzed ATP with a Km of 0.34 mM for Ca^{2+} ATPase and 0.48 mM for Mg^{2+} ATPase activity. Various nucloside triphosphate such as ITP, CTP, GTP, and UTP were also hydrolyzed by the enzyme. The enzyme was activated by Ca^{2+} and Mg^{2+} with Ka values of 1.47 mM and 2.51 mM, respectively; other divalent cations also activated the enzyme but to a lesser extent. Divalent cations like Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+} were potent inhibitors of the enzyme activity in the presence of Ca^{2+} or Mg^{2+}. Na^{+} plus K^{+} or HCO_3^- did not stimulate the Ca^{2+}/Mg^{2+} ATPase; the pH optimum was 8.5. The enzyme was insensitive to ouabain, verapamil, vanadate, oligomycin, N,N'-dicyclohexylcarbodiimide and NaN_3, but was markedly inhibited by 20 uM of gramacidin S and 50 uM of trifluoperazine. These properties of the purified Ca^{2+}/Mg^{2+} ATPase were similar to those of the Ca^{2+}/Mg^{2+} ATPase in plasma membrane and thus the purified Ca^{2+}/Mg^{2+} ATPase of heart plasma membrane appears to be in its native conformation.

Analysis of the purified Ca^{2+}/Mg^{2+} ATPase from the rat heart plasma membrane revealed the presence of 17 amino acids where leucine, glutamic acid and aspartic acid were major components and histidine, cycteine and methionine were minor components. CNBr peptide map of the enzyme showed only three fragments and this is consistant with the finding that the Ca^{2+}/Mg^{2+} ATPase had a relatively small content of methionine. The purified enzyme was associated with 19.7 umol phospholipid/mg protein which was 60 times higher than the phospholipid content in sarcolemma. The cholesterol content in the purified enzyme preparation was 0.75 umol/mg and this represented an 8-fold enrichment over sarcolemma. The membrane bound Ca^{2+}/Mg^{2+} ATPase and the purified enzyme were inhibited by phospholipase A and C but not by phospholipase D. The periodic acid-Schiff staining of the sodium dodecyl sulfate electrophoresis gel of purified Ca^{2+}/Mg^{2+} ATPase was positive. The polysaccharide content was enriched 8-fold in purified enzyme over plasma membrane; neuraminidase treatment decreased the polysaccharide content as well as the Ca^{2+}/Mg^{2+} ATPase activities. Concanavalin A prevented the ATP-dependent inactivation of the purified Ca^{2+}/Mg^{2+} ATPase and was found to bind to the purified enzyme with a K_D of 576 nM and Bmax of 4.52 nmol/mg protein. Ca^{2+} was also found to bind with Ca^{2+}/Mg^{2+} ATPase with a K_D of 0.384 mM and a Bmax of 1.85 umol/mg protein; Ni^{2+}, Mn^{2+}, Zn^{2+} at 1 mM inhibited the Ca^{2+} binding but Mg^{2+} and verapamil were without effect. Phospholipase A and neuraminidase decreased the Ca^{2+} binding by 20 - 30%; this indicated that Ca^{2+} binding with the purified enzyme may be partly due to the phospholipids and sialic acid residues associated with the enzyme. The purified Ca^{2+}/Mg^{2+}

ATPase was found to bind ATP-r-^{35}S with two affinities; the K_D values were 50.9 ± 0.8 and 1160 ± 198 nM and the Bmax values were 8.71 ± 0.16 and 145 ± 9.7 nmol/mg protein for high and low affinity sites, respectively. These results indicate that the purified Ca^{2+}/Mg^{2+} ATPase is a glycoprotein and requires phospholipds for its activity. Furthermore, this study suggests that Ca^{2+}/Mg^{2+} ATPase in the cell membrane is a Ca^{2+} binding protein which may serve as an ATP receptor.

A. STATEMENT OF THE PROBLEM

It has been demonstrated that a divalent cation dependent ATPase (Ca^{2+}/Mg^{2+} ATPase), which is activated in the presence of millimolar concentrations of Ca^{2+} or Mg^{2+}, is present in the heart plasma membrane (Dhalla and Zhao, 1989; Dhalla et al., 1982; Anand et al., 1977; McNamara et al., 1974; Malouf and Meissner, 1980). This Ca^{2+}/Mg^{2+} ATPase has been shown to be different from the Ca^{2+} stimulated ATPase of the sarcolemma, which is activated by micromolar concentrations of Ca^{2+}. It has been suggested that Ca^{2+}/Mg^{2+} ATPase may serve as a biochemical correlate of the electrophysiologically defined Ca^{2+} channels in heart sarcolemma (Dhalla and Zhao, 1989; Dhalla et al., 1982; Dhalla et al. 1977a; Dhalla et al., 1984; Dhalla et al., 1985a). Furthermore, it has been indicated that this enzyme is an ecto-ATPase and thus may participate in the degradation of the extracellular ATP, which has been shown to activate a Ca^{2+} channel in arterial smooth muscle cells and to induce Ca^{2+} transients in cardiac myocytes (Benham and Tsien, 1986; De Young and Scarpa, 1987). However, in order to elucidate the exact physiological role of the Ca^{2+}/Mg^{2+} ATPase in heart sarcolemma, it is important to purify the enzyme in its native form. Although Ca^{2+}/Mg^{2+} ATPases has been purified from the rabbit skeletal muscle transverse tubules (Hidalgo et al., 1983; Kirly, 1988), human oat cell plasma membranes (Knowles and leng, 1984) and sheep kidney medulla plasma membranes (Gantzer and Grisham,1979a,b), several discrepancies exist regarding the physical and biochemical characteristics of these ATPases (Dhalla and Zhao, 1989).

Earlier studies have shown that a Ca^{2+}-dependent ATPase, which is activated by millimolar concentrations of Ca^{2+} but not by Mg^{2+}, can be solubilized from rat heart sarcolemmal membranes upon digestion with trypsin, while the remaining Ca^{2+}/Mg^{2+} ATPase in the membrane after trypsin treatment can be solubilized with Triton X-100 and ultrasonication (Dhalla et al., 1981; Tuana and Dhalla, 1987). Both Ca^{2+}-dependent ATPase and Ca^{2+}/Mg^{2+} ATPase from the heart sarcolemmal membrane were subsequently purified (Tuana and Dhalla, 1982; Tuana and Dhalla 1988). It should be pointed out here that the heart membrane preparation employed in these studies was heavy sarcolemmal fraction which was isolated by low-speed centrifugation. This sarcolemmal fraction contained plasma membrane, the basement membrane and other cell surface material (Dhalla and Pierce, 1984; Moffat et al., 1983). Since the previous methods for solubilizing the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase involved the treatment of trypsin, it is not clear whether the isolated enzyme is an intact enzyme or an active fragment of the holoenzyme. On the other hand, the light sarcolemmal membrane fraction, which sediments only at high centrifugal force, exhibited 10-15 times higher activity of Ca^{2+}/Mg^{2+} ATPase in comparison to that of heavy sarcolemmal preparation (Zhao and Dhalla, 1988; Zhao et al., 1989). Furthermore, Ca^{2+}-dependent ATPase was not released upon treating the light sarcolemmal preparation with trypsin (Zhao and Dhalla, 1988). It was therefore thought worthwhile to undertake a study for characterizing the membrane-bound Ca^{2+}/Mg^{2+} ATPase in the light sarcolemmal preparation. In addition, it is planned to solubilize, purify and characterize the Ca^{2+}/Mg^{2+} ATPase from rat heart light plasma membrane fractions. The characterization of the purified Ca^{2+}/Mg^{2+} ATPase includes the behaviour of the enzyme activity under a wide variety of experimental conditions as well as determination of the chemical composition and ligand binding activities. In view of the importance of membrane phospholipids in regulating Ca^{2+}-related processes (Langer, 1978; Philipson, 1980), it is proposed to investigate the phospholipid requirement for the purified Ca^{2+}/Mg^{2+} ATPase with respect to enzyme activity and Ca^{2+} binding property. It is hoped that the study will provide further information for elucidating the physiological function of Ca^{2+}/Mg^{2+} ATPase in the heart plasma membrane.

B. REVIEW OF THE LITERATURE

I. Introduction

During the past two decades a great deal of progress has been made for the understanding of Na^{+}-K^{+} ATPase (Na^{+}-K^{+} pump) and Ca^{2+}- stimulated ATPase (Ca^{2+} pump), which are known to be present in the heart cell membrane (Carafoli, 1987; Rothstein, 1968; Stekhoven and Bonting, 1981). These ATPases have been demonstrated to function as cation transport systems and require MgATP as a substrate. Another class of the ATPases, Ca^{2+}/Mg^{2+} ATPase, has also been found to exist in the heart sarcolemmal membrane, which is activated by millimolar concentrations of either Ca^{2+} or Mg^{2+} and utilizes ATP as a substrate. However, its exact physiological function is not clear at present. Although Ca^{2+}/Mg^{2+} ATPase has been shown to be different from the Ca^{2+}-stimulated ATPase, it is not clear whether Ca^{2+}/Mg^{2+} ATPases from different species or tissues belong to the same family. Nontheless the Ca^{2+}/Mg^{2+} ATPase has been localized to be present in the cardiac cell membrane. Upon differential centrifugation, Ca^{2+}/Mg^{2+} ATPase was found to appear in fractions enriched with cell membrane marker activities (Anand et al., 1977; Zhao and Dhalla, 1988). Furthermore, Ca^{2+} ATPase or Mg^{2+} ATPase was localized on the plasma membrane of the heart by employing cytochemical techniques (Malouf and Meissner, 1980). It is also not known whether more than one kind of Ca^{2+}/Mg^{2+} ATPases exist in the same cell. In fact it is difficult to state with certainty whether Ca^{2+} ATPase and Mg^{2+} ATPase activities are the expression of a single enzyme.

Several lines of evidence suggest that both Ca^{2+} ATPase and Mg^{2+} ATPase activities are due to the presence of a same enzyme in the cell membrane. Both activities were enriched similarly in the sarcolemmal fractions from heart muscle by differential centrifugation and sucrose density gradient methods (Zhao and Dhalla, 1988). Ca^{2+} ATPase and Mg^{2+} ATPase in heart sarcolemmal preparations exhibited similar pH optima (Zhao and Dhalla, 1988). Similar substrate specificity was found for Ca^{2+} ATPase and Mg^{2+} ATPase from different sarcolemmal preparations (Anand-Srivastava and Dhalla, 1987a; Zhao and Dhalla, 1988). The Ca^{2+} ATPase and Mg^{2+} ATPase activities were not additive (Dhalla et al., 1976a). The activities of Ca^{2+} ATPase and Mg^{2+} ATPase were depressed similarly by various inhibitors (Zhao and Dhalla, 1988). Furthermore, the heart membrane Ca^{2+} ATPase and Mg^{2+} ATPase activities were inactivated similarly by ATP (Zhao and Dhalla, 1988). The activities of Ca^{2+} ATPase and Mg^{2+} ATPase in plasma membranes from heart were decreased by detergents to the same extent (Zhao and Dhalla, 1988). In the case of solubilization study, detergents Triton X-100, digitonin, lysophosphatidylcholine and CHAPS produced a parallel solubilization of Ca^{2+} ATPase and Mg^{2+} ATPase activities from rat heart (Tuana and Dhalla, 1987; Tuana and Dhalla, 1988; Zhao and Dhalla, 1987b).

Various other studies on Ca^{2+}/Mg^{2+} ATPase from heart sarcolemma (Anand-Srivastava et al., 1982; Anand-Srivastava and Dhalla, 1987b; Panagia et al, 1982) have provided evidence that Ca^{2+} ATPase and Mg^{2+} ATPase may be two separate enzymes. Mg^{2+}

ATPase from heart sarcolemma was more sensitive to ADP and less sensitive to Pi inhibition in comparison to Ca^{2+} ATPase. Storage of sarcolemma at about $0^{o}C$ showed a greater increase in ATP hydrolysis with Ca^{2+} than with Mg^{2+}. The inhibitory effect of Mn^{2+} on Ca^{2+} ATPase was more than that on Mg^{2+} ATPase. Treatment of membranes with SDS or deoxycholate produced a greater reduction in Mg^{2+} ATPase than in Ca^{2+} ATPase (Anand-Srivastava et al., 1982). Further work on the effects of deoxycholate on Ca^{2+}/Mg^{2+} ATPase showed that in deoxycholate treated preparations, the Km value for Ca^{2+} ATPase was decreased whereas that for Mg^{2+} ATPase was increased. The Ka value for Mg^{2+} was not altered but Ka for Ca^{2+} was significantly reduced upon treating the sarcolemmal preparation with detergents (Panagia et al., 1982). A Ca^{2+} ATPase, which was not activated by Mg^{2+}, was solubilized by trypsin treatment from heart sarcolemma and purified to homogeneity (Dhalla et al., 1981; Tuana and Dhalla, 1982), whereas a substantial amount of Ca^{2+}/Mg^{2+} ATPase, which was activated by either Ca^{2+} or Mg^{2+}, was still present in the trypsin-treated membranes (Dhalla et al., 1981). On the other hand, Ca^{2+}/Mg^{2+} ATPase in another heart sarcolemmal preparation isolated by the sucrose density gradient method was not affected by trypsin treatment and no Ca^{2+} ATPase was released by trypsin (Zhao and Dhalla, 1988). Accordingly, it was suggested that two kinds of divalent cation activated ATPase were present in the heart sarcolemmal membrane. One of these was activated by Ca^{2+} and was solubilized by trypsin, while the other was activated by either Ca^{2+} or Mg^{2+} and was not affected by trypsin (Dhalla et al., 1981).

II. Differences between Ca^{2+}/Mg^{2+} ATPase and other ATP hydrolyzing enzymes

Besides the cell membrane Ca^{2+}/Mg^{2+} ATPase, there are other ATPases in various subcellular organelles which require Ca^{2+} and/or Mg^{2+} for their ATP hydrolyzing activities. It is thus necessary to exclude the possibility that the cell membrane Ca^{2+}/Mg^{2+} ATPase was a contaminant from other organelles (DePierre and Karnovsky, 1973; Dhalla and Pierce, 1984). Since extensive efforts have been made to distinguish Ca^{2+}/Mg^{2+} ATPase of heart sarcolemma from other ATPases, it is proposed to discuss these differences. It is now well known that Na^+-K^+ ATPase is a marker enzyme for the plasma membrane and requires Mg^{2+} for its activity. Both Na^+-K^+ ATPase and Mg^{2+} ATPase were separated during the purification of the Na^+-pump enzyme (Stekhoven and Bonting, 1981). Ouabain, a specific inhibitor of Na^+-K^+ ATPase, does not inhibit the Mg^{2+} ATPase of plasma membranes from heart (Tuana and Dhalla, 1988; Zhao and Dhalla, 1988). Ca^{2+}/Mg^{2+} ATPase was also insensitive to orthovanadate, a potent inhibitor of Na^+-K^+ ATPase (Zhao and Dhalla, 1988).

There is confusion in the terminology of Ca^{2+}-stimulated ATPase and Ca^{2+}/Mg^{2+} ATPase because the two enzymes often share common short names such as Ca^{2+} ATPase, Ca^{2+}-Mg^{2+} ATPase, Ca^{2+}-dependent ATPase and the usage of these names has been quite arbitrary. The Ca^{2+}-stimulated ATPase, which serves as a Ca^{2+} pump in the cell membrane, requires micromolar concentrations of Ca^{2+} and utilizes MgATP as a substrate. Although it is difficult, if not impossible, to distinguish papers on Ca^{2+}/Mg^{2+} ATPase from those on Ca^{2+}-stimulated ATPase without verifying the assay medium for the measurement of enzyme activity, the two enzymes are relatively easy to differentiate experimentally because Ca^{2+}/Mg^{2+} ATPase does not require the second divalent cation for its activity and, unlike Ca^{2+}-stimulated ATPase, it is not sensitive to orthovanadate (Zhao and Dhalla, 1988). Mitochondrial ATPase (F_1F_0 ATPase) is activated under the assay conditions similar to those for the cell membrane Ca^{2+}/Mg^{2+} ATPase (Penefsky et al., 1960; Pullman et al., 1960). Furthermore, mitochondrial inner membrane (submitochondrial particles), on which mitochondrial ATPase resides, is considered to be a common source of contamination in the cell membrane preparations. Therefore, it is important to establish the extent of contribution of the mitochondrial ATPase in the Ca^{2+}/Mg^{2+} ATPase activity in the cell membrane. By comparing the specific activities of marker enzymes of submitochondrial particles in the cell membrane preparation and in the purified submitochondrial preparation, it is possible to estimate the percentage of contamination in the cell membrane preparation assuming that no mitochondrial protein was selectively separated from the submitochondrial particles during the isolation procedures. The most commonly used marker enzymes for mitochondrial inner membrane are cytochrome c oxidase and succinic dehydrogenase (Dhalla and Pierce, 1984). Another approach to differentiate sarcolemmal Ca^{2+}/Mg^{2+} ATPase from mitochondrial ATPase is to demonstrate that Ca^{2+}/Mg^{2+} ATPase is not depressed by specific inhibitors of mitochondrial ATPase, like oligomycin, DCCD and NaN_3. Although sarcolemmal Ca^{2+}/Mg^{2+} ATPase is also depressed by the well known mitochondrial ATPase inhibitors, the concentrations of inhibitors were rather high. NaN_3 is a most often used specific inhibitor of mitochondrial ATPase with a Ki of about 10 uM; however, at this concentration NaN_3 did not inhibit Ca^{2+}/Mg^{2+} ATPase of rat heart sarcolemma. Kinetic studies with the sarcolemmal Ca^{2+}/Mg^{2+} ATPase showed that the Ki of NaN_3 for the enzyme was 10 mM (Zhao and Dhalla, 1988). It should be pointed out that even at high concentration of NaN_3, the sarcolemmal Ca^{2+}/Mg^{2+} ATPase was not inhibited by more than 20%.

Alkaline phosphatase is a non-specific phosphatase, which may also split the ^[b^\-phosphate group from ATP molecule. The Ca^{2+}/Mg^{2+} ATPase activity in sarcolemmal preparations can not be attributed to alkaline phosphatase for the following reasons: (i) Plasma membrane bound alkaline phosphatase exhibited an optimal activity at pH 10.3 and was almost inactive at pH 7.5 (Kwan et al., 1979), while Ca^{2+}/Mg^{2+} ATPase is usually assayed in pH 7.4-7.5. (ii) Under conditions for the assay of Mg^{2+} ATPase or Ca^{2+} ATPase, virtually no pNPP, an alkaline phosphatase substrate, was hydrolysed (Zhao and Dhalla, 1988). (iii) Alkaline phosphatase did not require Mg^{2+} or Ca^{2+} for its activity (Kwan et al., 1979). Therefore, any alkaline phosphatase activity during Ca^{2+}/Mg^{2+} ATPase assay is usually regarded as the non-specific hydrolysis of ATP and thus is subtracted from total activity. (iv) Vanadate strongly inhibited alkaline phosphatase (Lopez et al., 1976) but had little effect on Ca^{2+}/Mg^{2+} ATPase activity (Zhao and Dhalla, 1988). It shoud be mentioned that the ATP hydrolyzing activity of the cell membrane may also be the expression of ATP diphosphohydrolase as this enzyme has been shown to catalyze the hydrolysis of triphosphonucleotides and diphosphonucleotides to yield nucleoside monophosphate and inorganic phosphate (Knowles et al., 1983). Although it is difficult at present to rule out the possibility that a part of the Ca^{2+}/Mg^{2+} activity in cell membrane preparations is due to ATP diphosphohydrolase, the activity of ATP diphosphohydrolase can be distinguished from Ca^{2+}/Mg^{2+} ATPase by two criteria (Lebel et al., 1980). Firstly, the ATP diphosphohydrolase has been shown to hydrolyze both nucleoside triphosphate and nucleoside diphosphate at equal rates while Ca^{2+}/Mg^{2+} ATPase hydrolyzes nucleoside diphosphate at a much lower rate in comparison to that for nucleoside triphosphate. Secondly, the ATP diphosphohydrolase is not stimulated by concanavalin A, which is capable of stimulating Ca^{2+}/Mg^{2+} ATPases from many cell membranes. Furthermore, 10-20 mM NaN_3 inhibited the ATP diphosphohydrolase more than 50%. Thus it appears that Ca^{2+}/Mg^{2+} ATPase in the cell membrane is distinctly different from other ATP hydrolyzing enzymes in the cell.

IV. Biochemical properties of Ca^{2+}/Mg^{2+} ATPase

In this section it is proposed to discuss the properties of the membrane bound Ca^{2+}/Mg^{2+} ATPase whereas the properties of the purified Ca^{2+}/Mg^{2+} ATPase will be discussed together with the purification procedures.

a. Substrate Specificity and ATP-dependent Inactivation.

The cell membrane Ca^{2+}/Mg^{2+} ATPase was found to be selective for nucleoside 5'-triphosphates as it hydrolyzed ATP, GTP, ITP, UTP and CTP at similar rates, although ATP was a preferred substrate over others (Anand-Srivastava et al., 1982; Zhao and

Dhalla, 1988). ADP was hydrolyzed only at less than 20% of the rate of ATP hydrolysis (Anand-Srivastava et al., 1982; Zhao and Dhalla, 1988), while AMP and pNPP were hydrolyzed at negligible rates (Anand-Srivastava et al., 1982; Zhao and Dhalla, 1988). The true substrate was considered to be the nucleotide-metal complex. A unique feature of the sarcolemmal Ca^{2+}/Mg^{2+} ATPase lies in the fact that the enzyme was inhibited by ATP in some membrane preparations. In rat heart muscle, ATP induced an inactivation of the enzyme (Zhao and Dhalla, 1988). The ATP hydrolysis by Ca^{2+}/Mg^{2+} ATPase was not linear with time, but decreased in a few minutes to a rate of less than 30% of that obtained during the first minute. This inhibition was not due to the accumulation of the reaction products of the enzyme because inclusion of an ATP-regenerating system or addition of ADP and inorganic phosphate did not alter the time courseof ATP hydrolysis. The Lineweaver-Burk plot of the data was linear and this also indicated no substrate inhibition. Other nucleoside 5'-triphosphates and ADP also induced a similar inactivation of the enzyme (Zhao and Dhalla, 1988). Such observations led to the proposal that Ca^{2+}/Mg^{2+} ATPase activity might be regulated by local concentrations of ATP in the form of activation and inactivation (Zhao and Dhalla, 1988).

b. Cation Requirements.

The Ca^{2+}/Mg^{2+} ATPase was activated by Ca^{2+}, Mg^{2+} and several other divalent cations but the other divalent cations were not as potent as Ca^{2+} or Mg^{2+}. The Ca^{2+}/Mg^{2+} ATPase was activated by Co^{2+} and Mn^{2+} in the place of Ca^{2+} or Mg^{2+} but the activity obtained with Co^{2+} or Mn^{2+} was 20-70% of that with Mg^{2+} or Ca^{2+} (Zhao and Dhalla, 1988; Beeler et al., 1985). Other divalent cations, such as Zn^{2+}, Ni^{2+}, Sr^{2+}, Ba^{2+} and Cu^{2+}, activated the enzyme with even lesser potency (Anand et al., 1977; Zhao and Dhalla, 1988; Beeler et al., 1985). When Ca^{2+}/Mg^{2+} ATPase was fully activated by Ca^{2+} or Mg^{2+}, divalent cations other than Ca^{2+} and Mg^{2+} inhibited the Ca^{2+}/Mg^{2+} ATPase. Hg^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}, which are less effective in stimulating the enzyme, inhibited the Ca^{2+}/Mg^{2+} ATPase activity by 70-90% whereas Co^{2+} and Mn^{2+}, which activated the enzyme moderately, inhibited the Ca^{2+}/Mg^{2+} ATPase only by 10-60% (Zhao and Dhalla, 1988).

Neither Na^{+} nor K^{+} alone stimulated the Ca^{2+}/Mg^{2+} ATPase activity in the presence of Mg^{2+} (Anand-Srivastava et al., 1982) and in fact these monovalent cations slightly inhibited the enzyme (Zhao and Dhalla, 1988). Trivalent cation La^{3+}, which is known to interfere with cellular processes requiring Ca^{2+}, inhibited the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase competitively (Anand et al., 1977; Takeo et al., 1979). The effect of changes in the H^{+} concentration has also been examined on the enzyme activity. The cell membrane Ca^{2+}/Mg^{2+} ATPase was found to have a broad pH optima (pH 7.5-8.5) (Zhao and Dhalla, 1988; Anand et al., 1977). This can be taken to mean that Ca^{2+}/Mg^{2+} ATPase may be operational over a wide range of alkaline pH changes which the cells may undergo but its activity may be depressed rather sharply under acidic conditions.

c. Affinity of Ca^{2+}/Mg^{2+} ATPase for Ca^{2+}, Mg^{2+} or ATP and Reaction Kinetics.

The reported values of Ka and Km for the Ca^{2+}/Mg^{2+} ATPase varied from 0.3 mM to 0.8 mM for Ca^{2+} and Mg^{2+} and from 0.2 to 1.1 mM for ATP, respectively. The Ca^{2+}/Mg^{2+} ATPase exhibited a low affinity for divalent cations, as this enzyme was fully activated at millimolar concentrations of Ca^{2+} or Mg^{+} (Anand et al., 1977; Zhao and Dhalla, 1988). Unlike Na^{+}-K^{+} ATPase and Ca^{2+}-stimulated ATPase where low affinity (Km = 10 - 500 uM) and high affinity (Km = 0.2 - 1 uM) sites for ATP have been demonstrated (Stekhoven and Bonting, 1981), sarcolemmal membrane-bound Ca^{2+}/Mg^{2+} ATPase was found to have only low affinity site for ATP (Km = 20 uM - 5.8 mM) (Anand-Srivastava et al., 1982; Zhao and Dhalla, 1988; Tuana and Dhalla, 1982).

d. Stimulators.

Several substances have been reported to stimulate the heart membrane Ca^{2+}/Mg^{2+} ATPase. The studies on the interaction of concanavalin A (Con A) and heart membrane Ca^{2+}/Mg^{2+} ATPase has helped to reveal the Ca^{2+}/Mg^{2+} ATPase reaction mechanisms as well as the regulation and function of the enzyme. Con A stimulated the sarcolemmal Ca^{2+}/Mg^{2+} ATPase (Zhao and Dhalla, 1988); the degree of stimulation was from 2 to 3 times of the control activity. This effect was considered specific because Con A did not stimulate other membrane ATPases like Na^{+}-K^{+} ATPase, Ca^{2+}-stimulated ATPase and mitochondrial ATPase (Zhao et al., 1989) and the effect was inhibited by α-methylmannoside, which is known to block the carbohydrate binding site of Con A. The stimulatory effect of Con A was dose dependent; maximal stimulation being found at Con A concentration of 10 - 50 ug/ml. Kinetic studies revealed that both apparent Km for Ca ATP or Mg ATP and Vmax of the enzyme were increased by 5 - 10 ug/ml Con A (Zhao et al., 1989). Although it seems probable that Con A exerts its effect on sarcolemmal membrane Ca^{2+}/Mg^{2+} ATPase through its carbohydrate binding site on the membrane, there is little information regarding the events following this binding. Con A was found to abolish ATP-dependent inactivation (Beeler et al., 1985; Zhao and Dhalla, 1988). Con A has also been shown to mimic the effect of insulin with respect to the stimulation of the Ca^{2+}/Mg^{2+} ATPase and cell metabolism (Jarett and Smith, 1974). Because Con A is known to cross-link membrane proteins like other agents such as glutaraldehyde, different lectins and antiserum against plasma membrane, it was proposed that Con A may prevent the dissociation of the enzyme protein complex which may be important for the Ca^{2+}/Mg^{2+} ATPase activity (Beeler et al., 1985). It is also likely that Con A stimulation may occur through a direct interaction with the Ca^{2+}/Mg^{2+} ATPase protein. In this context Con A may either impede the binding of ATP at the regulatory site of the enzyme or block the transmission of events from the regulatory site to the processes occurring at the catalytic site and thus interfere with the communication between the regulatory and catalytic sites (Moulton et al., 1986). Since Con A is also known to affect the membrane fluidity, it is also possible that the augmentation of the Ca^{2+}/Mg^{2+} ATPase by Con A may be due to its action on the fluidity of cell membrane (Zhao et al., 1989). It seems that the sequence of adding ATP and Con A to the assay medium was important for demonstrating the effect of Con A on Ca^{2+}/Mg^{2+} ATPase. If ATP was present before Con A addition, Con A was unable to stimulate the Ca^{2+}/Mg^{2+} ATPase (Zhao and Dhalla, 1988); this indicated that once ATP was bound to the enzyme, Con A cannot reverse the process induced by ATP binding. The studies on the interaction of ATP and Con A with cell membrane Ca^{2+}/Mg^{2+} ATPase lead to a proposal that the Ca^{2+}/Mg^{2+} ATPase may be regulated by local concentrations of ATP whereas a lectin-like protein, which may be present in the cell membrane, may modulate the regulatory effect of ATP on this enzyme (Zhao and Dhalla, 1988). In this regard, it should be noted that endogenous lectins have been isolated from skeletal muscle (Beyer et al., 1980).

Other stimulators of Ca^{2+}/Mg^{2+} ATPase have not been studied as extensively as Con A. Cyclic AMP, which serves as a second messenger, is known to activate a protein kinase, phosphorylate different proteins and thus may modulate the enzyme activities. It was found that the rat heart sarcolemmal membrane phosphorylation in the presence of both cyclic AMP and protein kinase was accompanied with an increase in Ca^{2+} ATPase and Mg^{2+} ATPase activities; the Vmax value of the Ca^{2+}/Mg^{2+} ATPase was increased while the Ka value was not altered (Ziegelhoffer et al., 1979; Ziegelhoffer et al, 1984). It was concluded that the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase may be regulated by cyclic AMP dependent cell processes. The ionphores such as FCCP, valinomycin, Ca^{2+} ionophore A23187, which are known to increase the ion transport ATPase activities by collapsing the ion gradient built up across the membrane by the ATPases, did not exert any effect on the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase (Zhao and Dhalla, 1988).

e. Inhibitors.

No specific and sensitive inhibitor of heart membrane Ca^{2+}/Mg^{2+} ATPase has yet been discovered. Ca^{2+}/Mg^{2+} ATPase was resistant to ouabain, orthovanadate, low concentrations of NaN_3 and oligomycin (Anand et al., 1977; Zhao and Dhalla, 1988). Recently, gramicidin S, but not gramicidin D, was found to inhibit heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase with IC_{50} of 3.4 uM (Zhao and Dhalla, 1989). It should be pointed out that gramicidin S is a cyclic decapeptide antibiotic produced by a strain of Bacillus brevis. It is a cationic amphiphilic peptide which is more closely related to tyrocidins in terms of its biological and chemical properties than to other gramidicins (Hunter and Schwartz, 1967). The inhibitory effect of gramicidin S was dose dependent; the maximum inhibition was more than 95%. The type of inhibition of the sarcolemmal Ca^{2+}/Mg^{2+} ATPase by gramicidin S was apparently uncompetitive and in fact the rate of ATP-dependent inactivation of the enzyme was increased by the this agent. Gramicidin S also inhibited the Ca^{2+}-stimulated ATPase of sarcolemma and sarcoplasmic reticulum, but had no action on Na^{+}-K^{+} ATPase, mitochondrial ATPase, and myofibrillar ATPase. Interestingly, gramicidin S at low concentrations inhibited the heart muscle contraction. Since it has been suggested that heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase may be involved in the gating mechanism for the entry of Ca^{2+} into the cell upon stimulation, the depressed effect of gramicidin S on muscle contraction was suggested to be due to the inhibition of this enzyme (Zhao and Dhalla, 1989).

Both NaN_3 and oligomycin, potent inhibitors of mitochondrial ATPase, were found to inhibit the cell membrane Ca^{2+}/Mg^{2+} ATPases of heart (Zhao and Dhalla, 1988). The effects of these agents were evident at high concentrations because at low concentrations, which markedly inhibited mitochondrial ATPase, these agents did not affect the enzyme (Zhao and Dhalla, 1988). The percentage of the enzyme inhibition by NaN_3 was about 20% (Zhao and Dhalla, 1988). High concentrations of oligomycin (5 - 30 ug/ml) also depressed the heart plasma membrane Ca^{2+}/Mg^{2+} ATPase by about 20% (Zhao and Dhalla, 1988); this inhibition can not be accounted for from mitochondrial contamination of the cell membrane fraction (Jarett and McKeel, 1970). Therefore, it appears that the inhibition of heart membrane Ca^{2+}/Mg^{2+} ATPase by high concentrations of sodium azide and oligomycin may not represent mitochondrial contamination in the heart sarcolemmal membrane preparation but may be the property of this enzyme system.

Ca^{2+}/Mg^{2+} ATPase of heart sarcolemma was inhibited by trifluoperazine (IC_{50} = 50 ug/ml) (Zhao and Dhalla, 1988); the inhibitory effects were apparent at higher concentrations than those required for calmodulin antagonism. Since trifluoperazine is a hydrophobic compound and has been shown to exert fluidizing effect on the cell membrane (Seeman, 1977), it is possible that this drug at high concentrations may affect the cell membrane Ca^{2+}/Mg^{2+} ATPase by altering the microenvironment of the enzyme. Ruthenium red, which is considered to inhibit the Ca^{2+}-stimulated ATPase with a certain degree of specificity (Stekhoven and Bonting, 1981), was reported to partially (14-30%) inhibit the cell membrane Ca^{2+}/Mg^{2+} ATPase from heart sarcolemma (Anand et al., 1977). Other inhibitors such as NaF and maleic anhydride have also been found to depress the sarcolemmal membrane Ca^{2+}/Mg^{2+} ATPase (Sulakhe and Dhalla, 1971; Anand et al., 1977).

Modification of the sulfhydryl groups of the sarcolemmal membrane Ca^{2+}/Mg^{2+} ATPase with sulfhydryl reagents inhibited the enzyme activity. Different reagents have been found to produce varying degrees of inhibitory effects; NEM was less effective than PCMB. Millimolar concentrations of NEM inhibited the enzyme activity by less than 60% (Sulakhe and Dhalla, 1971; Tuana and Dhalla, 1982). PCMB inhibited the Ca^{2+}/Mg^{2+} ATPase at concentrations less than 1 mM (Anand et al., 1977; Zhao and Dhalla, 1988), but the Ca^{2+}/Mg^{2+} ATPases from hamster heart (Coetzee and Gevers, 1977) were not inhibited by PCMB. The differences in the effects of NEM and PCMB were not due to any difference in the permeability of these two drugs across the membrane because NEM was able to penetrate the cell membrane and should have been more effective than PCMB (Pearson et al., 1980).

f. Effect of Membrane Modification and Lipid Involvement.

Different phospholipases are known to hydrolyse different membrane phospholipids specifically. In view of the specificity of their action and the generally mild conditions under which the treatment can be carried out, phospholipases are often used to modify selectively the membrane structure by elimination or alteration of a specific group of phospholipids. Phospholipase A from Naja Naja, which degrades glycerophospholipids to lyso-derivatives and free fatty acids, was observed to reduce activity of heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase to 20-30 % of the control value (Anand-Srivastava and Dhalla, 1987). The Ca^{2+} ATPase was less sensitive to the phospholipase treatment than Mg^{2+} ATPase and the reduction in the enzyme activity by the phospholipase A treatment was associated with a decrease in the Vmax value without any changes in the Ka value (Anand-srivastava and Dhalla, 1987). Phospholipases C from C. Welchii, which hydrolyzes phosphatidylcholine and sphingomyelin, and from Bacillus cereus, which hydrolyzes phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine and produces diacylglycerol, inhibited the Mg^{2+} ATPase from heart sarcolemma (Anand-Srivastava and Dhalla, 1987). The phospholipase C-induced inhibition may also be due to the formation of phosphatidic acid, which was found to inhibit Mg^{2+} ATPase and was rapidly formed after phospholipase C treatment as a result of phosphorylation of diacylglycerol (Gandhi and Ross, 1986). Furthermore, phospholipase D from cabbage, which hydrolyzes the phospholipid into phosphatidic acid, also inhibited the cell membrane Mg^{2+} ATPase (Anand-Srivastava and Dhalla, 1987).

It should be pointed out that phosphatidylethanolamine N-methylation, which is an enzymic reaction consisting of a sequential addition of three methyl groups to the amino moiety of the phospholipid to form phosphatidylcholine, has also been implicated in regulating membrane-related events. The phosphatidylethanolamine N-methylation stimulated Ca^{2+}- stimulated ATPase of heart sarcolemma without any change in the Mg^{2+} ATPase (Panagia et al., 1986), but decreased Ca^{2+} ATPase activity in heart sarcolemma (Panagia et al., 1987). The alterations in membrane lipid contents are known to induce changes in the membrane fluidity and it has been long recognized that membrane fluidity changes influence membrane ATPase activities (Farias et al., 1975). Saturated phospholipid fatty acid and cholesterol reduced the fluidity of the lipid matrix of the cell membrane (Farias et al., 1975), while unsaturated fatty acid increased the fluidity of the membrane (Leoni et al., 1982). The Arrhenius plot of Ca^{2+}/Mg^{2+} ATPase has also been used extensively to study the influence of the lipids on the membrane bound enzyme. Breaks in the temperature dependence curve of the enzyme are thought to be due to mainly the phase changes in the lipid moiety, but other reasons might apply as well (Farias et al., 1975). There are reports which indicated no transition temperature for Ca^{2+}/Mg^{2+} ATPase (Zhao and Dhalla, 1988; Beeler et al., 1985). The Q_{10} of the ATP hydrolysis reaction was 2.1 - 4 (Forrester and Williams, 1977; Moulton et al., 1986). The apparent energy of activation varied from 3.3K - 11.5K for Mg^{2+} ATPase and 14K for Ca^{2+} ATPase (Beeler et al., 1985; Zhao and Dhalla, 1988).

Detergents or other membrane perturbing agents have been widely employed to disturb the membrane structure or to delipidate the membrane. Heart plasma membrane Ca^{2+}/Mg^{2+} ATPase was inactivated by the detergent treatments (Panagia et al., 1982; Anand-Srivastava et al., 1982; Beeler et al., 1985; Zhao and Dhalla, 1988). Ca^{2+}/Mg^{2+} ATPase activities from heart sarcolemma were decreased by the deoxycholate treatment; the depression of Mg^{2+} ATPase was greater than that of Ca^{2+} ATPase. The Km value for Ca^{2+} ATPase was decreased whereas that for Mg^{2+} ATPase was increased. Furthermore, the Ka value for Ca^{2+} ATPase was drastically reduced but remained unchanged for Mg^{2+} ATPase(Panagia et al., 1982). The mechanism by which detergents inhibit the

Ca^{2+}/Mg^{2+} ATPase is not clear; however, various detergents were found to increase the inactivation rate of cell membrane Ca^{2+}/Mg^{2+} ATPase but exerted relatively mild effect on the initial rate of ATP hydrolysis by the enzyme (Beeler et al., 1985; Zhao and Dhalla, 1988). Since different detergents, ionic or nonionic, were found to produce qualitatively similar inhibition, it is possible that this effect may not be due to the removal of specific phospholipids which may be essential for the enzyme function, but may be due to the disruption of the membrane structure as a result of non-specific lipid removal or detergent insertion. The fact that no exogenous phospholipids were able to restore the depression in Ca^{2+}/Mg^{2+} ATPase activity by the detergent (unpublished data from this laboratory) also supports this view. It is interesting to note that Con A, which does not interact with any specific phospholipids, prevented the inactivation of the enzyme by the detergents (Beeler et al., 1985; Zhao and Dhalla, 1988). It is also possible that the observed changes in the Ca^{2+}/Mg^{2+} ATPase activity upon disrupting the membrane may be due to conformational changes in the enzyme protein. In this regard it was observed that increasing concentrations of Ca^{2+} or Mg^{2+} within the range of 0.1 - 10 mM was found to lower gradually the α-helix content of cardiac sarcolemmal protein in the absence of ATP. On the other hand, the same concentrations of Ca^{2+} or Mg^{2+} activated the Ca^{2+}/Mg^{2+} ATPase stepwise in the presence of ATP. A quantitative relationship was found between Ca^{2+}- or Mg^{2+}-induced stimulation of the cardiac sarcolemmal Ca^{2+}/Mg^{2+} ATPase activity and diminution of the α-

helix content of membrane proteins in cardiac sarcolemma (Vrbjar et al., 1985a; Vrbjar et al., 1985b).

V. Purification and properties of the purified Ca^{2+}/Mg^{2+} ATPase

The properties of the Ca^{2+}/Mg^{2+} ATPase discussed earlier were related to the enzyme present in the isolated cell membrane preparations, which contains complex proteins, lipids and carbohydrates. The use of purified enzyme may thus eliminate the interference of other membrane components, at least proteins, for the expression of enzyme activities and can be seen to provide a clearer picture of the characteristics of the Ca^{2+}/Mg^{2+} ATPase. However, there are disadvantages in this approach because a membrane-bound protein such as Ca^{2+}/Mg^{2+} ATPase often requires harsh treatments for its solubilization and further purification. This may alter the vital microenvironment of the enzyme and thus may change the properties of the Ca^{2+}/Mg^{2+} ATPase. Since the cell membrane Ca^{2+}/Mg^{2+} ATPase was sensitive to detergents, it became very difficult to solubilize the enzyme in an active form. There are no common rules regarding the selection of detergents, except that mild detergents, especially nonionic and zwitterionic detergents, were considered more effective in solubilizing the active Ca^{2+}/Mg^{2+} ATPase. Other considerations taken to optimize the solubilization included the concentration of the detergents, the detergent to membrane protein ratio, pH, ion strength of the solubilizing medium, use of metal ion chelator (e.g. EDTA), cofactors such as Ca^{2+}, Mg^{2+} and ATP, proteinase inhibitor and protein stabilizing agents such as DTT, sucrose, and glycerol. Digitonin and a mixture of lysophosphatidylcholine and CHAPS were found effective in solubilizing the Ca^{2+}/Mg^{2+} ATPase from heart sarcolemma. High concentrations of NaI improved the solubilization of the enzyme by detergents. EDTA was also beneficial but Ca^{2+} or Mg^{2+} had no effect on the solubilization (Zhao et al., 1987b). Trypsin digestion was shown to solubilize a Ca^{2+}-dependent ATPase from heart sarcolemma, which was activated by Ca^{2+} only (Elimban et al., 1987; Tuana and Dhalla, 1982; Dhalla et al., 1981), and another ATPase, which was activated by either Ca^{2+} or Mg^{2+}, was solubilized from trypsin treated sarcolemma by sonicating the membranes with Triton X-100 (Tuana and Dhalla, 1987).

The Ca^{2+}-dependent ATPase solubilized from rat heart sarcolemmal membranes upon digestion with trypsin was purified by high speed centrifugation, ammonium sulfate fractionation, DEAE cellulose and Sepharose-6B column chromatography (Tuana and Dhalla, 1982). The purified protein was seen as a single protein band in nondenaturing polyacrylamide gel electrophoresis. In SDS-acrylamide gels, the enzyme dissociated into two subunits or fragments with molecular weights of about 55K and 12K. The molecular weight of the enzyme, estimated by gel filtration, was found to be about 67K. The enzyme utilized ATP with a Km of 0.20-0.26 mM, and was also able to hydrolyze ITP, CTP, GTP and ADP but at much lower rates. It was activated by Ca^{2+} with a Ka of 0.13 - 0.21 mM and by other cations in the order $Ca^{2+} > Mn^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$. Divalent cations like Ca^{2+}, Ni^{2+}, and Mg^{2+} were potent inhibitors. The enzyme was insensitive to ouabain, verapamil, oligomycin, cyanide and vanadate but was markedly inhibited by N-ethylmaleimide. Calmodulin failed to stimulate

Ca^{2+}-dependent ATPase but instead inhibited it slightly. Unlike K^+, Na^+ produced a marked inhibition of the enzyme activity, and this inhibition was associated with an 8- to 10-fold decrease in the affinity of the enzyme for Ca^{2+}. The competitive action of Na^+ indicated that the Ca^{2+}-dependent ATPase may be a site of Na^+-Ca^{2+} antagonism in the cell membrane (Tuana and Dhalla, 1982).

The remaining Ca^{2+}/Mg^{2+} ATPase in the rat heart sarcolemma after trypsin treatment was solubilized with Triton X-100 and purified by high speed centrifugation, ammonium sulfate fractionation, hydrophobic chromatography and gel filtration (Tuana and Dhalla, 1988). The purified protein was seen as a single band in nondenaturing polyacrylamide gel electrophoresis and its molecular weight by gel filtration was found to be about 240K. The SDS gel analysis showed three major and two minor protein bands with molecular weights of 90K, 80K, 67K, 20K, and 10K. Since the membranes were treated with trypsin, it is likely that the polypeptide composition of the purified Ca^{2+}/Mg^{2+} ATPase was due to proteolysis of the enzyme molecule itself, or 80K, 67K, 20K and 10K peptides were cleavage products of the 90K peptides. The enzyme utilized CaATP or MgATP as substrate with high affinity sites (Km=0.12-0.16 mM) and low affinity sites (Km=1 mM). The enzyme also utilized CTP, GTP, ITP, UTP, and ADP as substrates but at a lower rate in comparison to ATP. The enzyme was activated by Ca^{2+} (Ka=0.4 mM), and Mg^{2+}(Ka=0.2 mM) as well as by other cations in the order $Ca^{2+} > Mg^{2+} > Mn^{2+} > Sr^{2+} > Ba^{2+} > Ni^{2+} > Cu^{2+}$. The ATPase activity in the presence of Ca^{2+} was markedly inhibited by Mg^{2+}, Mn^{2+}, Ni^{2+}, and Cu^{2+} whereas the monovalent cations such as

Na^+ and K^+ were without effect. The enzyme did not exhibit Ca^{2+}-stimulated ATPase activity and was insensitive to calmodulin, ouabain, verapamil, D-600, oligomycin, azide and vanadate. Optimal pH for the enzyme activity was 8.5-9.0. The dog heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase, solubilized by trypsin treatment, Triton X-100 and sonication was subjected to a purification procedure similar to that of rat heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase (Tuana and Dhalla, 1987). This enzyme from the dog heart was labile when purified; the Ka values of the partially purified Ca^{2+}/Mg^{2+} ATPase were 0.34 mM for Ca^{2+} and 0.22 mM for Mg^{2+}. The Ca^{2+}/Mg^{2+} ATPase did not show any cross-reactivity with dog heart myosin antiserum, nor with Na^+-K^+ ATPase antiserum (Tuana et al., 1984; Tuana and Dhalla, 1987).

V . Changes in Ca^{2+}/Mg^{2+} ATPase activities due to various interventions.

The cardiac cell membrane Ca^{2+}/Mg^{2+} ATPase was affected when the animals, heart or membrane preparations were subjected to certain physiological, pharmacological or pathological interventions. The studies in these areas are considered to enhance our understanding of the regulation of this enzyme as well as physiological role of this enzyme in heart function.

a. Physiological and Pharmacological Aspects.

The rat heart sarcolemmal Mg^{2+} ATPase of 10-day-old rats was significantly higher than that of adult rats (Vornanen, 1984); the Ka value for Mg^{2+} was also higher in 10-day-old rats. On the other hand, no significant differences were found in Ca^{2+} ATPase activities between 10-day-old and adult rats. Since heart function is increased during development of the animals, it appears that Ca^{2+}-related enzyme activity in the sarcolemmal membrane may not play a functional role. On the other hand, in vitro studies on heart muscle contractility and heart sarcolemmal Ca^{2+} ATPase activity showed that the contractile force development by the perfused heart exhibited a positive corelation with sarcolemmal Ca^{2+} ATPase activity in the presence of different concentrations of calcium (Dhalla et al., 1982). La^{3+} inhibited both contractile force development of perfused heart and sarcolemmal Ca^{2+} ATPase activity; a linear corelation was found between contractile force and Ca^{2+} ATPase activity in the presence of different concentrations of La^{3+} (Dhalla et al., 1982). Several agents, such as propranolol (Dhalla et al., 1977b), quinidine, procaine amide, lidocaine (Dhalla et al., 1978b), and gramicidine S (Zhao and Dhalla, 1989), which inhibited the Ca^{2+}/Mg^{2+} ATPase activity of heart sarcolemma, also inhibited the contractile force of perfused heart. Divalent cations, such as Co^{2+}, Ni^{2+}, and Mn^{2+}, which depressed Ca^{2+} currents in the myocardium, were also found to depress both Ca^{2+}/Mg^{2+} ATPase activity of heart sarcolemma and the contractile force of isolated heart (Harrow et al., 1978). Such a corelation between heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase activity and heart muscle contractility during various pharmacological intervention suggests that the enzyme may play an important role in the processes heart muscle contraction.

b. Pathological Aspects.

The cell membrane Ca^{2+}/Mg^{2+} ATPase activities from heart have been found to be altered under various pathological conditions.

Such changes may or may not associate with the changes in other membrane functions such as Na^+-K^+ ATPase, Ca^{2+}-stimulated ATPase and Ca^{2+}-binding, indicating a specific relationship between certain pathological events and cell membrane Ca^{2+}/Mg^{2+} ATPase alterations. The most detailed work on this aspect was carried out on the heart tissue for defining the clinical significance of changes in Ca^{2+}/Mg^{2+} ATPase.

In a stable model of cardiac hypertrophy without any signs of congestive heart failure, the sarcolemmal Ca^{2+} ATPase activity was significantly higher (Heyliger and Dhalla, 1986); Lineweaver-Burk plot revealed that the Ka value of the ATPase for Ca^{2+} was significantly lower whereas the Vmax value did not change in hypertrophied hearts. The increased sarcolemmal Ca^{2+} ATPase, which is considered to be involved in Ca^{2+} gating mechanism (Dhalla et al., 1977a), may partly explain the hyperfunction of the hypertrophied heart. It should be pointed out that congestive heart failure is a final stage of many heart diseases in which impaired cellular Ca^{2+} and energy metabolism as well as changes in sarcolemmal Ca^{2+}/Mg^{2+} ATPase have been commonly observed (Dhalla et al., 1978a). In failing hearts due to mitral insufficiency, sarcolemmal Mg^{2+} ATPase in both ventricles was found to increase after six months (Prasad et al., 1985) and this change was associated with an increase in the Na^+-K^+ ATPase of left ventricle (Prasad et al., 1985; Khatter and Prasad, 1976). However, prazosin treatment of animals with heart failure did not affect the Mg^{2+} ATPase activity but prevented the increase of Na^+-K^+ ATPase (Prasad et al., 1985). A strain of Syrian hamster is known to develop congestive heart failure due to a genetically determined myopathy where cardiac damage was progressive in nature during the course of the disease and a significant depression in sarcolemmal Ca^{2+} ATPase or Mg^{2+} ATPase was observed at late stage of the cardiomyopathy (Panagia et al., 1984; Dhalla et al., 1976). Among the observed changes in phospholipid composition of the diseased heart sarcolemma, lysophosphatidylcholine was markedly increased; this phospholipid was shown to inhibit the Ca^{2+}/mg^{2+} ATPase in heart sarcolemma (Zhao and Dhalla, 1988).

Compromised cardiac function in chronic diabetes was found to be associated with elevated sarcolemmal Ca^{2+}/Mg^{2+} ATPase activity and depressed Na^{+}-K^{+} ATPase activity (Pierce and Dhalla, 1983; Dhalla et al., 1985; Borda et al., 1988). Since cholesterol content of the sarcolemmal membranes was significantly increased in diabetic heart, this change has been implicated in causing the observed increment of the sarcolemmal Ca^{2+}/Mg^{2+} ATPase (Pierce and Dhalla, 1983). This change in the enzyme activity may be limited to heart because the activity of human red blood cell membrane Mg^{2+} ATPase from diabetic subjects has been reported to be about 60 % of the normal group (Adamson et al., 1986). The sarcolemmal Ca^{2+}/Mg^{2+} ATPase activity was found to first increase and then decrease upon feeding high cholesterol diet to rats (Moffat and Dhalla, 1985). Experimental hypothyroidism has also been shown to be associated with the depression in myocardial contractile function but the depressed activity of Na^{+}-K^{+} ATPase in the hypothyroid heart was not associated with any changes in the Ca^{2+}/Mg^{2+} ATPase activity (Daly et al., 1986). On the other hand, positive end-expiratory pressure (PEEP) treatment was found to induce a decrease in cardiac output and the PEEP plasma depressed the heart sarcolemmal Ca^{2+} ATPase activity as well as cardiac contraction (Utsunomiya et al., 1982).

VI. Proposed physiological functions for Ca^{2+}/Mg^{2+} ATPase

Despite extensive studies and various attempts over the past several years, the exact physiological function of the cell membrane Ca^{2+}/Mg^{2+} ATPase is far from clear. The importance of the enzyme in the cell membrane function can be appreciated from the facts that the Ca^{2+}/Mg^{2+} ATPase is widely distributed among animal species and divergent tissues, and that this ATPase has often been shown to have the highest ATPase activity in the cell membrane; the activity of Ca^{2+}/Mg^{2+} ATPase is sometimes 10 to 20 times higher than that of Na^{+}-K^{+} ATPase or Ca^{2+}-stimulated ATPase.

a. Gating Mechanism for Ca^{2+}-Channel in Heart Muscle.

Although depolarization of the cardiac muscle is known to open Ca^{2+}-channels in the cell membrane and permit the entry of Ca^{2+} along the concentration gradient, it is not clear how exactly these Ca^{2+} channels open by an electrical stimulus and how these channels close upon the disappearance of the electrical impulse. Since opening of Ca^{2+} channels can be conceived to involve the physical movements of proteins, it was suggested that this process is a highly regulated event in which Ca^{2+}/Mg^{2+} ATPase may be involved for opening Ca^{2+} gates in the sarcolemmal membrane (Dhalla et al., 1985; Dhalla et al., 1984; Dhalla et al., 1982; Dhalla et al., 1977b). It is pointed out that opening of the slow channels through which the inward Ca^{2+} current traverses the heart sarcolemma has been shown to require metabolic energy in the form of ATP (Sperelakis and Schneider, 1976). If this is the case then an ATPase system similar to the sarcolemmal Ca^{2+}/Mg^{2+} ATPase can be readily seen to be involved in opening the Ca^{2+} channels in myocardium. It is noteworthy that several energy yielding agents including ATP have been shown to produce an increase in contractile force of the cardiac muscle (Antoni et al., 1960; Saks et al., 1978). Thus, it appears that Ca^{2+}/Mg^{2+} ATPase may represent a biochemical correlate of the electrophysiologically defined Ca^{2+}-channels in heart sarcolemma and this enzyme may be intimately involved in opening these gates by phosphorylating these proteins in the cell membrane. This should not be viewed as a phenomenon analogous to the active Ca^{2+}-transport because the movement of Ca^{2+} from the extracellular fluid into the cell is a passive process but it is the opening of Ca^{2+} channels that requires phosphorylation through the participation of the sarcolemmal Ca^{2+}/Mg^{2+} ATPase system.

The proposal that Ca^{2+}/Mg^{2+} ATPase is involved in the Ca^{2+} influx is based on several biochemical and pharmacological observations. The activation of Ca^{2+}/Mg^{2+} ATPase by Ca^{2+} was shown to exhibit a linear correlation with contractile force development in the myocardium (Dhalla et al., 1982). Kinetic studies on the enzyme indicated that maximal ATP hydrolysis occurs in the presence of 3-4 mM Ca^{2+} with 4 mM ATP (Anand et al., 1977) and since the extracellular concentration of Ca^{2+} is 1.25 mM, an enzyme system implemented in Ca^{2+} influx would have to be stimulated at millimolar concentrations. In addition, phosphorylation of sarcolemma, which is associated with increased slow channel activity, was found to stimulate the Ca^{2+} ATPase activity of heart sarcolemma (Ziegelhoffer et al., 1979). Drugs such as propranolol, quinidine, lidocaine, and procainamide, which depressed Ca^{2+} influx, also decrease Ca^{2+} ATPase activity (Dhalla et al., 1977b; Dhalla et al., 1978b). Cations such as Co^{2+}, Ni^{2+}, and Mn^{2+} that block the slow inward Ca^{2+} current depressed the activity of Ca^{2+} ATPase (Harrow et al., 1978). If the function of Ca^{2+}/Mg^{2+} ATPase was to open Ca^{2+}-channels in the sarcolemmal membrane, then a decrease in its activity will depress Ca^{2+} entry and contractile force development. On the other hand, a prolonged increase in Ca^{2+}/Mg^{2+} ATPase activity will favor an increase in Ca^{2+} influx for the occurrence of intracellular Ca^{2+} overload and subsequent myocardial cell damage. Such changes in the sarcolemmal Ca^{2+}/Mg^{2+} ATPase activity have been observed in different types of models for cardiac contractile failure (Heyliger and Dhalla, 1986; Dhalla et al., 1976b; Dhalla et al., 1978a; Bing, 1983). Extracellular ATP has been reported to activate membrane Ca^{2+} channels in neurons (Yatani et al., 1978) and induce Ca^{2+} transients in cardiac myocytes (DeYoung and Scarpa, 1987). Furthermore, electrical stimulation of the rat heart sarcolemmal membranes _in vitro_ was found to increase Ca^{2+} ATPase activity; this effect was depressed by Ca^{2+}-antagonists, verapamil and D600, but not other types of inhibitors such as propranolol and ouabain. These experiments further support the view that the Ca^{2+}/Mg^{2+} ATPase may be involved in the gating mechanism for opening Ca^{2+} channels (Ziegelhoffer and Dhalla, 1987; Ziegelhoffer et al., 1984). Since this enzyme was inhibited by Na^+ competitively, it was suggested to be involved in Na^+-sensitive Ca^{2+}-entry into the cardiac cell (Elimban et al., 1987;

Tuana and Dhalla, 1982). Although such studies are encouraging in defining the physiological role of Ca^{2+}/Mg^{2+} ATPase in heart function, extensive research is needed to demonstrate that this enzyme serves as a gating mechanism for opening Ca^{2+} channels in the sarcolemmal membrane.

b. Regulation of ATP Levels in the Extracellular Compartment.

The role of intracellular ATP as the most immediate source of energy has been known for many years while the importance of extracellular ATP in regulating various biological processes, such as platelet aggregation, vascular tone, neurotransmission, cell membrane permeability, cell nutrition, cell volume control, cell Ca^{2+} transients, cardiac function and muscle contraction, has been recognized recently (Artalejo and Garcia-Sancho, 1988; Forrester and Williams, 1977; Rorive and Kleinzeller, 1972; Trams, 1974; Gordon, 1986; Dhalla et al., 1982). It should be noted that ATP is the principal active substance released from the purinergic nerve cells and is packed and released as a cotransmitter with noradrenalin, 5'-hydroxytryptamine, and acetylcholine (Nagy et al., 1983). The ATP has also been shown to be released from synaptic vesicles (Pearson et al., 1980; Gordon, 1986), vascular endothelial cells and smooth muscle cells (Pearson et al., 1980; Pearson and Gordon, 1979), and heart cells (Forrester and Williams, 1977). Various mammalian cells have been shown to be capable of synthesizing ATP on their outer surface (Agren et al, 1971). Furthermore, several ecto-enzymes, like glyceralaldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, adenylate kinase and creatine phosphokinase, have also been shown capable of synthesizing ATP extracellularly (Fenselau and Long, 1974; Packham et al., 1974; Saks et al, 1977). The extracellular ATP and/or ATP bound in the membrane can be seen to serve as a substrate for the sarcolemmal ATPase. The extracellular ATP can be degraded by ectonucleotidases, namely ATPase, ADPase and 5'-nucleotidase, which sequentially catabolize ATP to ADP, AMP and finally adenosine which has a wide variety of biological effects. Although ectonucleotidases on the endothelial cells are responsible for the removal of plasma ATP, these enzymes are not restricted to endothelial cells but have a wide spread distribution amongst tissues (Panagia et al., 1984; Forrester and Williams, 1977). The ecto-ATPase may also provide a mechanism for the termination of the ATP signal in processes where ATP may be acting as a neurotransmitter (Carraway et al., 1980; Dhalla et al., 1982; Burnstock, 1981). It has been indicated that selective release of a very small portion of ATP from myocardial or vascular cells with catabolism to adenosine by ectonucleotidases can account for the amounts of adenosine found in blood and thus may exert a powerful vasodilation in response to hypoxia. It has also been suggested that the ecto Ca^{2+} ATPase may be related to the detection of extracellular ATP as a chemotactic agent (DePierre and Karnovsky, 1974a; DePierre and Karnovsky, 1974b). The contractile nature of the enzyme may help the cell membrane to contract and thus Ca^{2+}/Mg^{2+} ATPase can been seen to help the cardiac cell maitain its shape and volumn. Accordingly, the Ca^{2+}/Mg^{2+} ATPase can be considered to function as a purinergic receptor on the cell membrane.

c. **Contractile Functions in Cell Membranes.**

It has been reported that fibrous proteins resembling muscle actomyosin are intrinsic components of the cell membrane (Barmann et al., 1986; Patel and Fairbanks, 1986; Matovcik, 1986), and these proteins are associated with Ca^{2+}-dependent ATPase activity (Elimban et al., 1987; Rosenthal et al., 1970). A Ca^{2+}-dependent ATPase purified from heart sarcolemma, which showed little Mg^{2+} ATPase activity, was compared with heart myosin ATPase. The sarcolemmal Ca^{2+}-dependent ATPase was found to differ from myosin ATPase because actin, a well known activator of myosin ATPase, failed to show any activation of Ca^{2+}-dependent ATPase. However, the purified sarcolemmal Ca^{2+}-dependent ATPase exhibited K^{+}-EDTA stimulated ATPase activity similar to that seen with myosin ATPase. This study suggested that Ca^{2+}-dependent ATPase of sarcolemma might be a myosin-like protein, or a part of such a protein (Elimban et al., 1987). The contractile nature of the enzyme may help the cell membrane to contract and thus Ca^{2+}/Mg^{2+} ATPase can been seen to help the cardiac cell maintain its shape and volume.

From the foregoing discussion, it is clear that the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase represents a unique type of ATPase in comparison to the commonly known transport ATPases in the cell membrane and other subcellular organelles. Because of its universal existence and generally high activity, the cell membrane Ca^{2+}/Mg^{2+} ATPase can be seen to play an important functional role in the cell. Although many suggestions have been made about the physiological function of the enzyme, the current evidence is circumstantial. Most of the studies on the properties of the membrane-bound Ca^{2+}/Mg^{2+} ATPase of heart have employed the heavy sarcolemmal preparations containing basement membrane and relatively little attention has been paid to the characterization of the enzyme in the purified plasma membrane from the heart. Furthermore much more work is required with the purified enzyme before we can fully appreciate the exact physiological role of the Ca^{2+}/Mg^{2+} ATPase in cellular function.

C. Experimental Pocedures

I. Materials

Sepharose 6B, nucleotides, lysophosphatidylcholine, digitonin, gramicidin S, trifluoperazine, sodium cholate, Triton X-100, octylglucoside, phenylmethylsulfonyl fluoride, N,N'-dicyclohexylcarbodiimide, vanadium pentoxide, N-ethylmaleimide, NaN_3, CHAPS, phospholipase A_2 (from Naja naja venom), C (from C. welchii) and D (from cabbage), neuraminidase, periodic acid, concanavalin A, polysaccharide, 1-ethyl-2-(3-(1-ethylnaphtho (1.2d)-thiazolin-2-ylidene-2-methylpropenyl) naphtho (1,2d)-thiazolium bromide, ATP and nitrendipine were purchased from Sigma Chemical Co. (St. Louis, MO). Adenosine 5'-O-(3-thiotriphosphate) (ATPrS) was obtained from Boehringer Mannheim as tetralithium salt. [^{35}S] ATPrS as triethylammonium salt, $^{45}CaCl_2$, [^{125}I] iodocancanavalin A, [^3H] prazosin, [^3H] dihydroalprenolol, and [^3H] PN200-110 were from New England Nuclear. Affi-Gel Blue, sodium dodecyl sulfate and acrylamide were from Bio Rad. All other chemicals were of reagent grade.

II. Isolation of heart plasma membranes

The plasma membranes were isolated from the rat heart by the sucrose density gradient method according to the method of Pitts (1979). Male Sprague-Dawley rats were decapitated and their hearts quickly removed and placed in ice-cold homogenizing medium (0.6M sucrose, 10 mM imidazole-HCl, pH 7.0). The ventricles were washed thoroughly, diced with a pair of scissors, homogenized with a polytron PT-20 (setting 5, 20 sec x 5 with 20 sec intervals) in homogenizing medium (3.5 ml/g tissue). The homogenate was centrifuged at 12,000 x g for 30 min. The supernatant was diluted with 5 ml/g KCl/MOPS buffer (160 mM KCl, 20 mM MOPS, pH 7.4) and centrifuged at 96,000 x g for 60 min. The pellets were resuspended in 1 ml/g KCl/MOPS buffer and the suspension was carefully laid over sucrose buffer (30% sucrose, 0.3 M KCl, 50 mM sodium pyrophosphate, 0.1 M Tris-HCl, pH 8.3). After centrifugation at 95,000 x g for 90 min, the white band at the suspension-sucrose interface was removed, diluted with 3 volume of KCl/MOPS buffer, and centrifuged at 96,000 x g for 30 min. The pellets were suspended in 50 mM Tris-HCl, pH 7.4 and employed as purified plasma membrane preparation. On the basis of extensive marker enzyme studies (Makino et al., 1987; Dixon et al., 1988), this fraction was found to be of sarcolemmal origin and to contain minimal (1-3%) cross contamination by other subcellular organelles.

III. Assay of ATPase activity

The Ca^{2+} or Mg^{2+} ATPase activity was assayed at 37^{o}C in 1 ml assay medium containing 50 mM Tris-HCl, pH 7.4, 4 mM Tris-ATP, and 4 mM $CaCl_2$ or $MgCl_2$. The final protein concentration for the purified Ca^{2+}/Mg^{2+} ATPase was 1 to 3 ug/ml whereas that for the membrane preparation was 20 to 30 ug/ml. The reaction was started by addition of ATP and stopped 1 min after by the addition of 0.5 ml 10% sodium dodecyl sulfate. Experimental conditions that differed from these described here are given in the text. The Ca^{2+} ATPase or Mg^{2+} ATPase activity was calculated as difference between the amount of Pi formed in the presence of 4 mM Ca^{2+} or Mg^{2+} and that in the absence of any divalent cation but with 1 mM EDTA. Inclusion of 0.1 mM EDTA in the reaction medium did not affect the measured ATPase activity. The estimation of Pi was carried out by the method of Taussky and Shorr (1953). The protein concentration was determined by the method of Lowry et al (1951) and Bio-Rad protein reagent.

IV. Solubilization and purification of Ca^{2+}/Mg^{2+} ATPase

Ca^{2+}/Mg^{2+} ATPase of rat heart plasma membrane was solubilized at a protein concentration of 10 mg/ml by a solubilizing medium composed of 5 mg/ml lysophosphatidylcholine, 5 mg/ml CHAPS, 0.6M NaI, 1mM EDTA, 50 mM Tris-HCl, pH 7.4, at room temperature for 10 min. Non-solubilized material was removed by centrifugation at 100,000 x g at 4^{o}C for 60 min. The supernatant was layered on a sucrose gradient (3 ml each of 15%, 20%, 25%, 30%, 35% and 40% sucrose in 50 mM Tris-HCl, pH 7.4) and centrifuged at 160,000 x g overnight (16 h). Fractions of 1 ml each were collected and those with Ca^{2+}/Mg^{2+} ATPase activity were pooled. The pooled sample was subjected to the blue dye ligand chromatography which had an affinity to the enzyme binding sites for adenine and ribose rings. A column (0.6 x 15 cm) was packed with Affi-Gel Blue and fractions from sucrose gradient exhibiting ATPase activity were applied to the column equilibrated with 50 mM Tris-HCl, pH 7.4. The protein was eluted from this column with the equilibrating buffer and the fractions (0.5 ml of each) with Ca^{2+}/Mg^{2+} ATPase activity were pooled and applied to sepharose 6B column. The Affi-Gel Blue column was then washed with 1M NaCl and/or 6M urea. Gel-filtration of the pooled fractions from the above column was performed on a Sepharose 6B column (1 x 20 cm) which had been equilibrated with 50 mM Tris-HCl, pH 7.4. The protein was eluted with the equilibrating buffer at the rate of 10 ml/h. The fractions (0.5 ml each) exhibiting ATPase activity obtained from this column were pooled, analyzed for purity and used for other studies. During the purification process, a great deal of lipid material remained associated with the enzyme and this was further evident from the fact that the enzyme activity eluted from gel filtration column near the void volume. This property made ligand binding studies relatively easy to perform because the enzyme can be rapidly separated from incubating media by filtration or centrifugation.

V. Determination of moleclar weight of the purified Ca^{2+}/Mg^{2+} ATPase

In order to determine the molecular weight of the enzyme, the lipids associated with Ca^{2+}/Mg^{2+} ATPase were removed by employing digitonin. For this study a purification procedure similar to the one described above was employed except that 1% digitonin was used to solubilize the sarcolemmal membrane and that same concentration of digitonin was included in the sucrose gradient and the equilibrating buffer of Affi-Gel Blue column and Sepharose 6B column. This yielded an enzyme with low specific activity but apparently the associated lipids were removed. The

Ca^{2+}/Mg^{2+} ATPase was applied to a Sepharose 6B column equilibrated with 1% digitonin and 50 mM Tris-HCl, pH 7.4, which had been calibrated with proteins with known molecular weights and the ratio of elution volume to void volume was determined. The subunits of Ca^{2+}/Mg^{2+} ATPase and their molecular weights were determined by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli (Laemmli, 1970). Protein samples were prepared by heating at 100^{o}C for 3 min in the presence of 1% sodium dedocyl sulfate, 0.06M Tris-HCl, pH 6.8, 5% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. Approximately 1 ug protein was applied to a polyacrylamide slab gel (140 x 160 x 1.5 mM) containing 4% stacking gel and 10% seperating gel. The electrophoresis was carried out at room temperature with a current of 20 mA till the dye passed through the stacking gel. The current was increased to 30 mA per slab as the dye moved to the separating gel. The gels were stained with silver stain (Morrissey, 1981). Standard proteins with known molecular weight (Sigma Dalton Mark VI, SDS-6) were also run simultanously.

VI. Amino acid composition and peptide mapping

Amino acid analysis was performed using a Beckman 6300 amino acid analyzer with ninhydrin detection. Performic acid oxidation plus HCl hydrolysis for cycteine was performed according to Hirs (1967). HCl hydrolysis with thioglycolic acid for tryptophan was performed according to Matsubara and Sasaki (1969). Peptide mapping was done by treating the enzyme with cyanogen bromide according to Nikodem and Fresco (1979).

VII. Lipid measurements

For measuring the phospholipid content, the plasma membrane preparation and the purified enzyme preparation were extracted with chloroform: methanol, 2:1 (vol: vol), and the phospholipids were separated by using two-directional thin-layer chromatography as described previously (Makino et al., 1987). Phospholipid phosphorus was measured as described elsewhere (Pierce et al., 1983). For the measurement of cholesterol content, the preparations were extracted with chloroform:methanol, 2:1 (vol:vol), and the cholesterol content was determined by high performance liquid chromatographic method using Beckman system Gold HPLC and a silica column.

VIII. Treatment with phospholipidases and neuraminidase

The purified Ca^{2+}/Mg^{2+} ATPase (5 ug/ml) and the plasma membrane preparation (1 mg/ml) were treated with phospholipases A, C or D for 30 min at room temperature in a medium containing 50 mM Tris-HCl, pH 7.4, 100 mM KCl and 1 mM $CaCl_2$ (Anand-Srivastava and Dhalla, 1987b). The concentration of phospholipidase A was 1.6 unit/ml, phospholipase C 1.0 unit/ml and phospholipase D 1.0 unit/ml. The neuraminidase treatment was performed by incubating the preparations in a medium containing 0.31 unit/ml neuraminidase, 50 mM Tris-HCl, pH 7.4, and 20 mM KCl at 30°C for 20 min (Zhao et al., 1989).

IX. Periodic acid-Schiff staining

The electrophoresis gel was stained with periodic acid-Schiff staining reagent according to the method of Segest and

Jackson, 1972. The molecular weight standard (Sigma Dalton Mark VI, SDS-6) was run simultanously and stained with coomassie brilliant blue.

X. Concanavalin A binding

Concanavalin A binding was measured according to the method employed previously (Zhao et al., 1989). Total binding was determined by incubating about 1 ug purified protein in a medium containing 0.05% bovine serum albumin, 50 mM Tris-HCl, pH 7.4, and various concentrations of [^{125}I] concanavalin A in a total volume of 0.2 ml at room temperature for 10 min. The reaction was stopped by adding 1 ml cold Tris-buffer. The tubes were centrifuged for 10 min and washed with cold buffer. The radioactivity was counted on a Beckman gamma counter. Nonspecific binding was measured by incubating the purified protein under the same condition but in the presence of 0.2 mM α-methylmannoside added before the addition of concanavalin A. Specific concanavalin A binding was calculated by subtracting the nonspecific binding from the total binding.

XI. Measurement of polysaccharides

Polysaccharide content of heart plasma membrane and purified Ca^{2+}/Mg^{2+} ATPase was measured by colorimetric method using 1-ethyl-2-(3-(1-ethylnaphtho (1.2d)-thiazolin-2-ylidene-2-methylpropenyl) naphtho (1.2d)-thiazolium bromide as dye reagent (Janda and Work, 1971). The standard used was polysaccharide from zoogloea ramigera. Sialic acid was measured according to Aminoff (1961).

XII. ATPrS binding to the purified Ca^{2+}/Mg^{2+} ATPase

For ATPrS binding assay, various concentrations of [^{35}S] ATPrS were incubated with about 0.5 ug purified protein in 0.5 ml medium containing 50 mM Tris-HCl, pH 7.5 at 37°C for 30 min. The ATPrS binding was terminated by filtering the incubating medium through Millipore filter (0.45 uM pore size) under vaccum suction. The filter was washed with 4 ml of ice cold water. The radioactivity on the filter was counted on a Beckman scintilation counter. The non-specific binding was determined by the same method but 4 mM ATP was included in incubating medium.

XIII. ATP-independent Ca^{2+} binding

ATP-independent Ca^{2+} binding activity of purified Ca^{2+}/Mg^{2+} ATPase was determined in the absence of Mg^{2+} or Na^{+} as described elsewhere (Pierce et al., 1983). The purified enzyme (2 ug) was incubated at 37°C for 5 min with various concentrations of $^{45}CaCl_2$ in Tris-HCl, pH 7.4 buffer. The binding was terminated by filtrating the incubation medium through Millipore filter (0.45 uM). The filter was washed with 4 ml of ice cold water. The non-specific binding measured in the absence of protein was subtracted from total binding.

XIV. Other measurements

ATP-dependent Ca^{2+} uptake (Ca^{2+} pump activity) was measured according to Makino et al. (1987). The binding of [^{3}H] prazosin, an α-adrenergic receptor antagonist, [^{3}H] dihydroalprenolol, a β-adrenergic receptor antagonist, and [^{3}H] PN200-110, a Ca^{2+} antagonist, was measured according to

Williams and Lefkowitz (1979), Mukherjee et al. (1979), and Wagner, et al. (1986), respectively.

XV. Statistical Analysis

At least 3 to 5 different preparations were employed for each observation and where appropriate the results were given as mean ± S.E.. Students' "t" test was used for determining the significance of the difference between control and experimental values.

D. RESULTS

I. Characterization of plasma membrane bound Ca^{2+}/Mg^{2+} ATPase

The plasma membrane preparation was found to hydrolyze ATP in the presence of Ca^{2+} or Mg^{2+}. The time course of the enzyme reaction (Table 1) revealed that the ATPase activities started decreasing 2 min after initiating the incubation with ATP. However, the reaction was linear with respect to the protein concentration in the range of 10-50 ug/ml. Accordingly, all experiments were carried out for 1 min by using 20-30 ug membrane protein in the incubation medium. In one set of experiments, the ATPase activities were measured by varying the concentrations of either Ca^{2+} or Mg^{2+} in the presence of 4 mM ATP whereas in another set of experiments, the concentration of ATP was varied in the presence of 4 mM Ca^{2+} or Mg^{2+}. Both Ca^{2+} ATPase and Mg^{2+} ATPase were fully activated in the presence of 4 mM Ca^{2+} or Mg^{2+}, respectively. The Lineweaver-Burk plot analysis of the data revealed Ka and Km values (Table 2) similar to those reported by employing heavy sarcolemmal preparations (Anand-Srivastava et al., 1982; McNamara et al., 1974; Panagia et al., 1982). However, the Vmax values for both Ca^{2+} ATPase and Mg^{2+} ATPase were 10 to 15 times higher than those reported earlier.

Other divalent cations also activated the ATPase but were much lesser effective than Ca^{2+} or Mg^{2+}(Table 3). However, in the presence of Ca^{2+} or Mg^{2+}, most of divalent cations were inhibitory; Zn^{2+}, Cd^{2+}, and Cu^{2+} were most inhibitory, while Ba^{2+} was least inhibitory (Table 4). The Mg^{2+} ATPase,

Table 1. Ca^{2+}/Mg^{2+} ATPase activities of heart plasma membrane at different times of incubation.

The Ca^{2+}/Mg^{2+} ATPase activities were determined in aliquots taken at different intervals after the addition of 4 mM ATP in a medium containing 4 mM Ca^{2+} or 4 mM Mg^{2+}. The results represent the rates of ATP hydrolysis during the time periods indicated. Each value is a mean ± SE of six experiments.

Time of incubation (min)	Ca^{2+} ATPase activity (umol Pi/mg/min)	Mg^{2+} ATPase activity (umol Pi/mg/min)
1	5.9 ± 0.9	7.6 ± 1.4
2	5.7 ± 0.8	6.7 ± 1.4
4	3.5 ± 0.4	3.5 ± 0.5
8	2.2 ± 0.1	2.1 ± 0.3
16	1.3 ± 0.3	0.9 ± 0.1

Table 2. Kinetic parameters of the rat heart plasma membrane Ca^{2+}/Mg^{2+} ATPase.

ATPase activities were determined by varying the concentration of ATP and keeping the concentrations of Ca^{2+} or Mg^{2+} at 4 mM as well as by varying the concentration of Ca^{2+} or Mg^{2+} and keeping the concentrations of ATP at 4 mM. The Lineweaver-Burk plots of the data revealed Km, Ka, and Vmax values. Each value is a mean ± SE of four experiments.

	Ca^{2+} ATPase	Mg^{2+} ATPase
A. Varying ATP concentrations		
Km for ATP (mM)	0.23 ± 0.03	0.33 ± 0.04
Vmax (umol Pi/mg/min)	7.03 ± 0.66	9.32 ± 0.64
B. Varying cation concentrations		
Ka for Ca^{2+} or Mg^{2+} (mM)	0.69 ± 0.40	0.71 ± 0.40
Vmax (umol Pi/mg/min)	6.73 ± 0.80	8.82 ± 0.53

Table 3. Divalent cation specificity for the ATP hydrolysis by rat heart plasma membrane.

Membranes were incubated in the presence of 4 mM ATP and 4 mM of different cations. The ATP hydrolysis due to the presence of each cation was expressed as a percentage of that in the presence of Mg^{2+}.

Cations	Relative ATP hydrolysis	Cations	Relative ATP hydrolysis
Mg^{2+}	100	Cd^{2+}	11
Ca^{2+}	75	Ni^{2+}	8
Co^{2+}	36	Zn^{2+}	5
Mn^{2+}	22	Ba^{2+}	0
Sr^{2+}	14	Cu^{2+}	0

Table 4. Effect of different ions on the rat heart plasma membrane Ca^{2+}/Mg^{2+} ATPase activities.

Membranes were incubated in the presence of 4 mM ATP and 4 mM of Ca^{2+} or Mg^{2+}, and the indicated concentrations of different ions. The ATPase activities are expressed as percentages of Ca^{2+} ATPase or Mg^{2+} ATPase in the absence of any other ions.

Ions added	Concentration (mM)	Relative Ca^{2+} ATPase activity	Relative Mg^{2+} ATPase activity
None	--	100	100
Mg^{2+}	4	133	102
Ba^{2+}	4	109	102
Mn^{2+}	4	41	30
Co^{2+}	4	38	30
Ni^{2+}	4	21	12
Cd^{2+}	4	17	9
Cu^{2+}	4	13	11
Zn^{2+}	4	10	1
Na^{+}	100	97	90
K^{+}	100	93	90
HCO_3^{-}	50	102	89
Vanadate	1	96	81

unlike Ca^{2+} ATPase, was slightly inhibited by 100 mM NaCl, 100 mM KCl, 50 mM $NaHCO_3$, or 1 mM vanadate.

In one series of experiments, the time course of ATP hydrolysis due to Ca^{2+} /Mg^{2+} ATPase was investigated (Fig. 1). We found that the rate of ATP hydrolysis by the Ca^{2+} /Mg^{2+} ATPase decayed exponentially. The first order rate constant of inactivation was 0.15 to 0.30 min^{-1} for Mg^{2+} ATPase activity and 0.14 to 0.18 min^{-1} for Ca^{2+} ATPase activity at 37 ^{o}C. The Ca^{2+} /Mg^{2+} ATPase activities were not further inactivated after they were reduced to 20 to 30% of initial ATP hydrolyzing rate. In the absence of ATP, the inactivation rate was about 150 times lower than that in the presence of ATP (Figs 1 and 2). Addition of 1 mM KH_2PO_4, ADP, and AMP in the assay medium did not show significant change in the initial rates of ATPase activities; similar results were obtained when the reaction was studied in the presence of AP_5A, a well-known inhibitor of adenylate kinase (Table 5). This indicated that the inactivation was not due to the product inhibition but was likely an ATP-dependent inactivation. Other high energy nucleotides like CTP, UTP, GTP, and ITP, which were hydrolyzed by the ATPase at a rate similar to that for ATP, also induced similar inactivation of the enzyme (Table 6). On the other hand, ADP was hydrolyzed at a lower rate in comparison to that for ATP but inactivated the enzyme at a higher rate (Table 6). It should be noted from Table 5 that ADP increased the inactivation rate of the enzyme due to ATP. pNPP was not

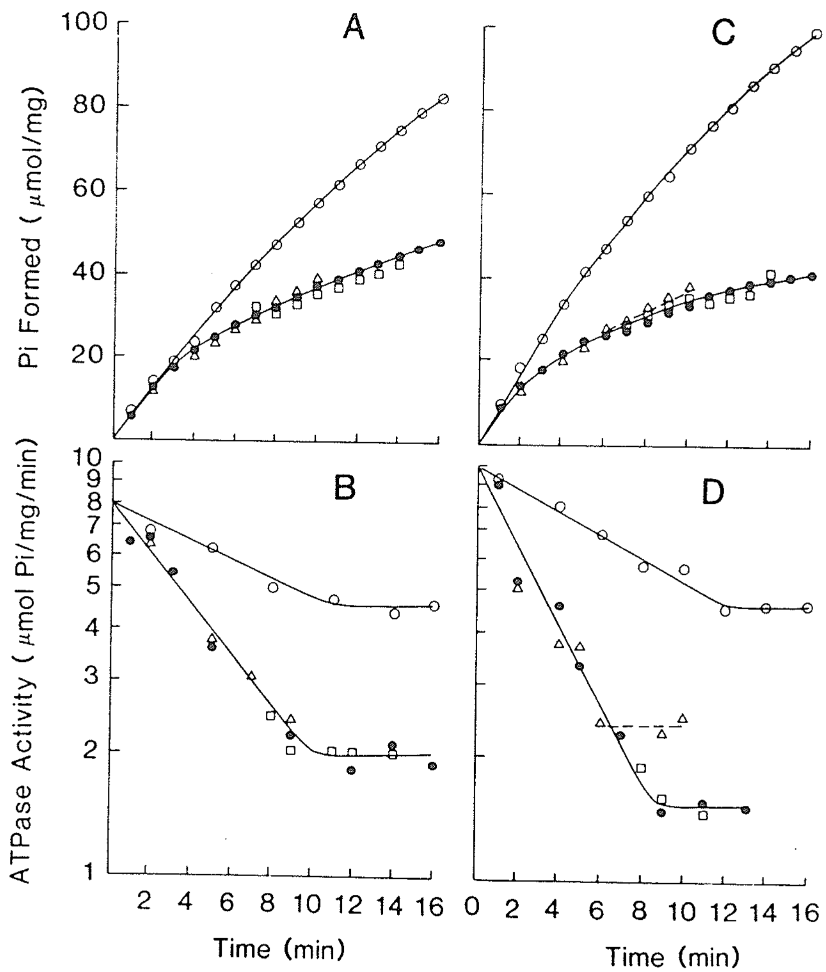

Fig. 1 Rates of ATP hydrolysis by the heart plasma membrane Ca^{2+}/Mg^{2+} ATPase. (A) Time course of ATP hydrolysis by Ca^{2+} ATPase in the absence (•) and presence of 20 ug/ml Con A added before (o) or 2 min (△) and 7 min (□) after the addition of ATP; (B) rate of ATP hydrolysis by Ca^{2+} ATPase calculated from the data in A; (C) time course of ATP hydrolysis by Mg^{2+} ATPase; (D) rate of ATP hydrolysis by Mg^{2+} ATPase in the absence (•) and presence of 20 ug/ml Con A added before (o) or 2 min (△) and 7 min (□) after the addition of ATP. The data for ATPase activity are plotted on a semilogarithmic graph.

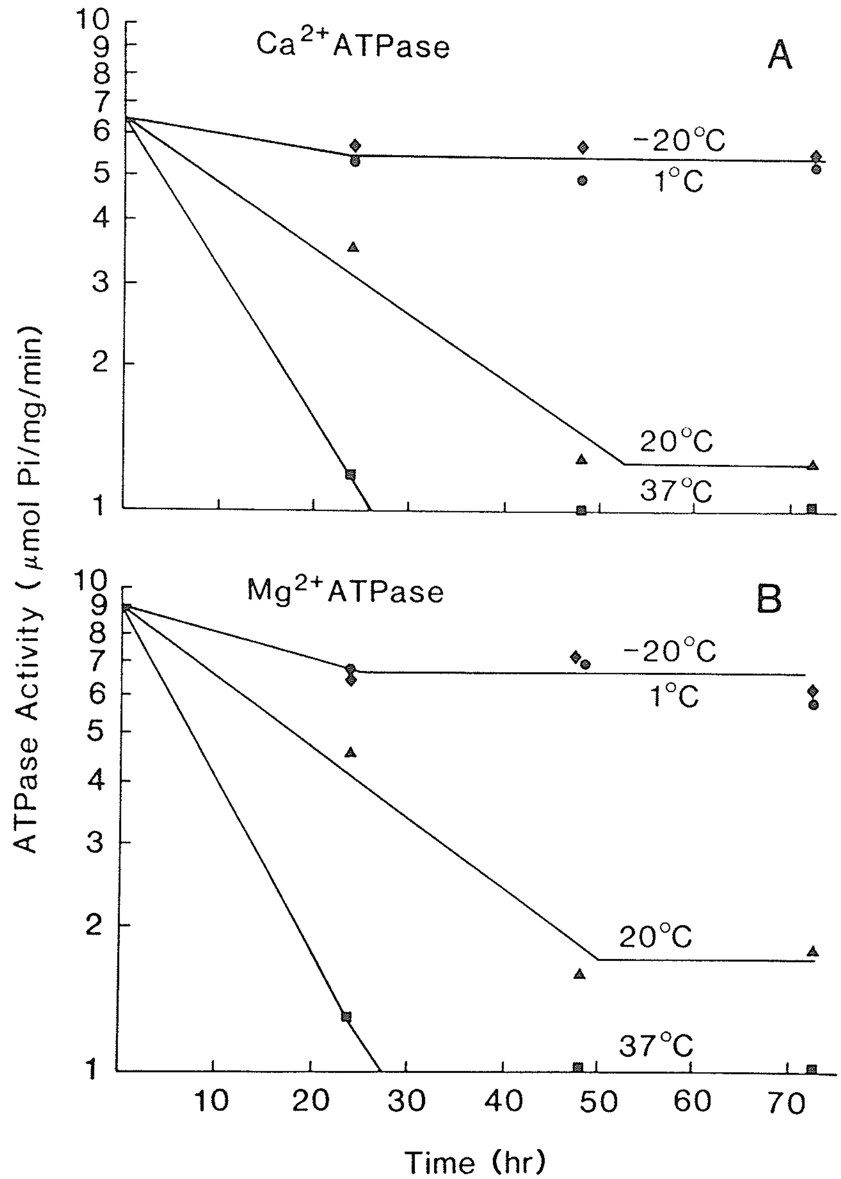

Fig. 2 Rate of inactivation of the heart plasma membrane Ca^{2+}/Mg^{2+} ATPase in the absence of ATP. The membranes (0.3 mg/ml) were incubated for 24 to 72 h at the indicated temperatures. Aliquots were removed at various times and the initial ATPase activities were measured at 37 C. The data are plotted on a semilogarithmic graph.

Table 5. Effects of ADP, AMP, and Pi on the heart plasma membrane Ca^{2+}/Mg^{2+} in the absence and presence of diadenosine pentaphosphate (AP_5A). ATPase activities were assayed in the absence or presence of various agents as indicated. KH_2PO_4 was used as Pi. The concentration of AP_5A was 10 uM.

	Without AP_5A		With AP_5A	
	Initial ATPase rate (umol Pi/mg/min)	Inactivation rate constant (min^{-1})	Initial ATPase rate (umol Pi/mg/min)	Inactivation rate constant (min^{-1})
Control:				
Ca^{2+} ATPase	6.7	0.15	5.2	0.15
Mg^{2+} ATPase	8.9	0.15	6.3	0.14
ADP (1 mM):				
Ca^{2+} ATPase	7.8	0.26	6.8	0.17
Mg^{2+} ATPase	9.2	0.31	9.0	0.20
AMP (1mM):				
Ca^{2+} ATPase	6.9	0.13	6.5	0.14
Mg^{2+} ATPase	8.7	0.17	8.3	0.14
Pi (1 mM):				
Ca^{2+} ATPase	7.5	0.14	4.8	0.15
Mg^{2+} ATPase	8.3	0.15	6.7	0.18
ADP (1 mM) + Pi (1 mM):				
Ca^{2+} ATPase	7.2	0.26	5.2	0.25
Mg^{2+} ATPase	8.4	0.26	6.5	0.31

Table 6. Substrate specificity and inactivation rate constants for the heart plasma membrane Ca^{2+}/Mg^{2+} ATPase.

Membranes were incubated in the assay medium containing 50 mM Tris-HCl, pH 7.5, and 4 mM of nucleotides.

Substrate	In the presence of 4 mM Ca^{2+}		In the presence of 4 mM Mg^{2+}	
	Hydrolysis rate (umol/mg/min)	Inactivation rate constant (min^{-1})	Hydrolysis rate (umol/mg/min)	Inactivation rate constant (min^{-1})
ATP	9.3	0.18	12.3	0.30
CTP	10.8	0.18	8.2	0.32
ITP	8.2	0.18	10.9	0.16
UTP	8.9	0.18	9.9	0.21
GTP	7.9	0.21	8.8	0.25
ADP	1.9	0.32	1.9	0.41

hydrolyzed under same conditions, indicating that the ATPase activity was not due to a non-specific phosphatase. After pretreating the plasma membrane with ATP for 30 min, when the ATPase activity was reduced to 20 to 30% of original ATP hydrolyzing rate, removal of ATP partially restored the enzyme activity; however, the inactivation rate was not influenced by ATP pretreatment (Table 7).

The ATP-dependent inactivation rate of the enzyme seems to be independent of temperature below 37^{o} C (Fig.3). Con A, a lectin, was found to prevent ATP-dependent inactivation partially (Fig.1). Pretreating the membrane with Con A did not alter the initial hydrolysis rate of the Ca^{2+} /Mg^{2+} ATPase, but reduced the ATP-dependent inactivation rate constant from 0.21 min^{-1} for Mg^{2+} ATPase and 0.14 min^{-1} for Ca^{2+} ATPase to 0.07 and 0.05 min^{-1}, respectively. The enzyme activities were also stabilized at a much higher level than that in the absence of Con A. However, Con A was found to interact with heart membrane Ca^{2+} /Mg^{2+} ATPase slowly because adding Con A 2 to 7 min after the addition of ATP failed to prevent the inactivation immediately (Fig.1). The effects of Con A on the inactivation of the enzyme were blocked completely by the presence of 50 mM α-methylmannoside; this indicates that Con A protects the enzyme by binding to oligosaccharide residues, which may form part of the enzyme molecule.

II. Solubilization and purification of Ca^{2+}/Mg^{2+} ATPase

The rat heart plasma membrane Ca^{2+}/Mg^{2+} ATPase activity was solubilized by lysophosphatidylcholine, and a mixture of

Table 7. Effect of washing on the heart plasma membrane Ca^{2+}/Mg^{2+} after pretreatment with ATP.

Membranes were incubated for 30 min at 22 °C in a medium containing 50 mM Tris-HCl, pH 7.5, 4 mM ATP, 4 mM $CaCl_2$ or $MgCl_2$. The activities of these membranes were markedly depressed (20 to 30% of the control). Control samples were incubated in medium without the ATP. The samples were then centrifuged at 100,000 g for 30 min and the pellet was thoroughly washed and resuspended in 50 mM Tris-HCl, pH 7.5, buffer and used for ATPase activity assay.

Preincubation medium	Initial rate of ATPase (umol/mg/min)		Inactivation rate constant of ATPase (min^{-1})	
	Ca^{2+} ATPase	Mg^{2+} ATPase	Ca^{2+} ATPase	Mg^{2+} ATPase
Control ($MgCl_2$)	6.3	7.5	0.21	0.21
$MgCl_2$ + ATP	5.2	5.5	0.23	0.21
Control ($CaCl_2$)	6.8	6.7	0.23	0.16
$CaCl_2$ + ATP	3.8	5.2	0.21	0.23

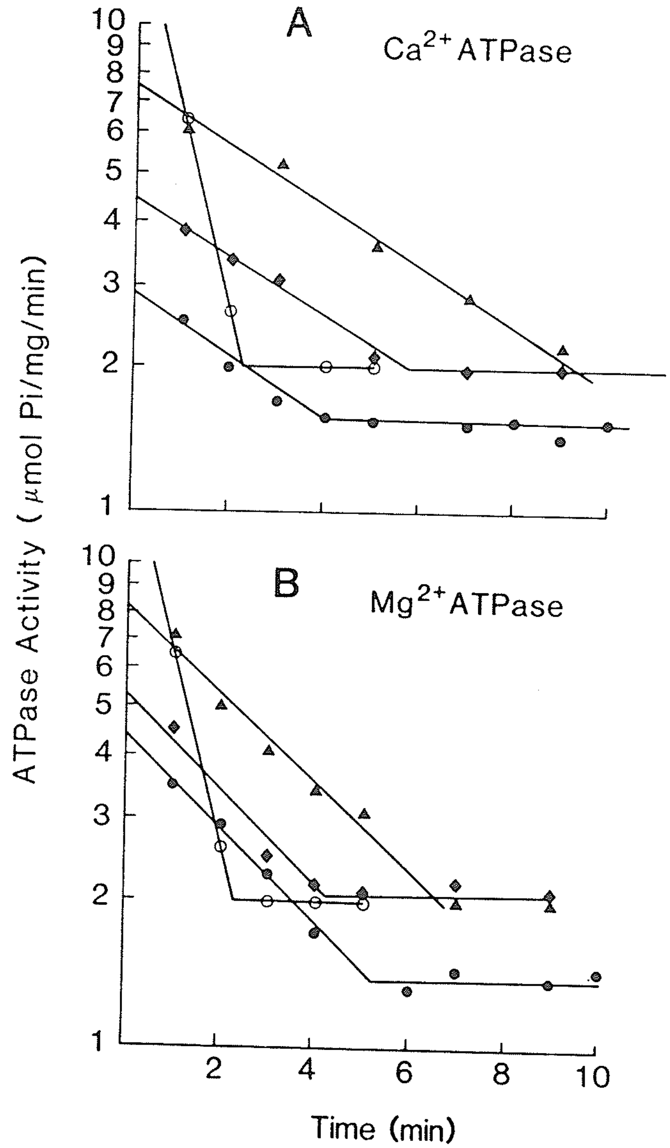

Fig. 3 Rate of inactivation of the heart plasma membrane Ca^{2+}/Mg^{2+} ATPase in the presence of ATP. ATPases were assayed at 22°C (•), 30°C (♦), 37°C (▲) and 43°C (○).

lysophosphatidylcholine and CHAPS; the yield of the solubilized enzyme was about 20% (Table 8). The Ca^{2+} ATPase activity and Mg^{2+} ATPase activity were solubilized to a similar extent. The effect of various concentrations of lysophosphatidylcholine and CHAPS in the solubilization of Ca^{2+}/Mg^{2+} ATPase is shown in Fig.4. The maximal solubilization for both Ca^{2+} ATPase and Mg^{2+} ATPase was seen at 5 mg/ml lysophosphatidylcholine plus 5 mg/ml CHAPS. It can be seen from the activities remaining in the pellet (Table 8) that some detergents like sodium cholate and Trition X-100 inactivated the Ca^{2+}/Mg^{2+} ATPase. While octylglucoside did not affect the enzyme activity, it failed to solubilize the enzyme. Higher concentrations of these detergents did not increase the yield of the solubilized enzyme (data not shown).

Sodium iodide, a chaotropic salt, disorders the structure of water, promotes the transfer of hydrophobic groups from an apolar environment to the aqueous phase and facilitates the solubilization of the enzyme. The effect of various concentrations of NaI on the solubilization of Ca^{2+}/Mg^{2+} ATPase is shown in Fig.4. 0.6-1.2 M NaI produced maximal increment in the solubilization by detergents. The yield of the enzyme activities in the supernatant was raised to about 40%. The pH of solubilizing medium had no significant effect on the solubilization of the Ca^{2+}/Mg^{2+} ATPase (data not shown). EDTA was found beneficial to the solubilization of the enzyme, while Ca^{2+}, Mg^{2+}, dithiothreitol, glycerol, and concanavalin A, which is a stimulator of Ca^{2+}/Mg^{2+} ATPase (Zhao et al.,

Table 8. Solubilization of rat heart plasma membrane Ca^{2+}/Mg^{2+} ATPase by detergents.

The solubilizing medium contained 50 mM Tris-HCl, pH 7.5, and various detergents. The control Ca^{2+} ATPase and Mg^{2+} ATPase activities of the plasma membrane were 6.6 and 9.1 umol Pi/mg/min, resepctively. Each value is an average of 6 experiments.

Detergents	ATPase activity (% of presolubilization control)			
	Ca^{2+} ATPase		Mg^{2+} ATPase	
	Supernatant	Pellet	Supernatnat	Pellet
Sodium cholate (10 mM)	2	1	1	1
Triton X-100 (1%)	10	2	13	2
CHAPS (1%)	11	53	7	39
Octylglucoside (30 mM)	7	87	9	89
Zwittergent 3-14 (0.3 mM)	4	44	6	36
Lysophosphatidylcholine (1%)	15	26	17	30
Lysophosphatidylcholine (1%) + CHAPS (0.5%)	22	27	21	22
Digitonin (1%)	21	62	19	55
Digitonin (0.5%) + CHAPS (0.5%)	22	47	21	45
Digitonin (0.5%) + lysophosphatidylcholine (0.5%)	20	50	18	48

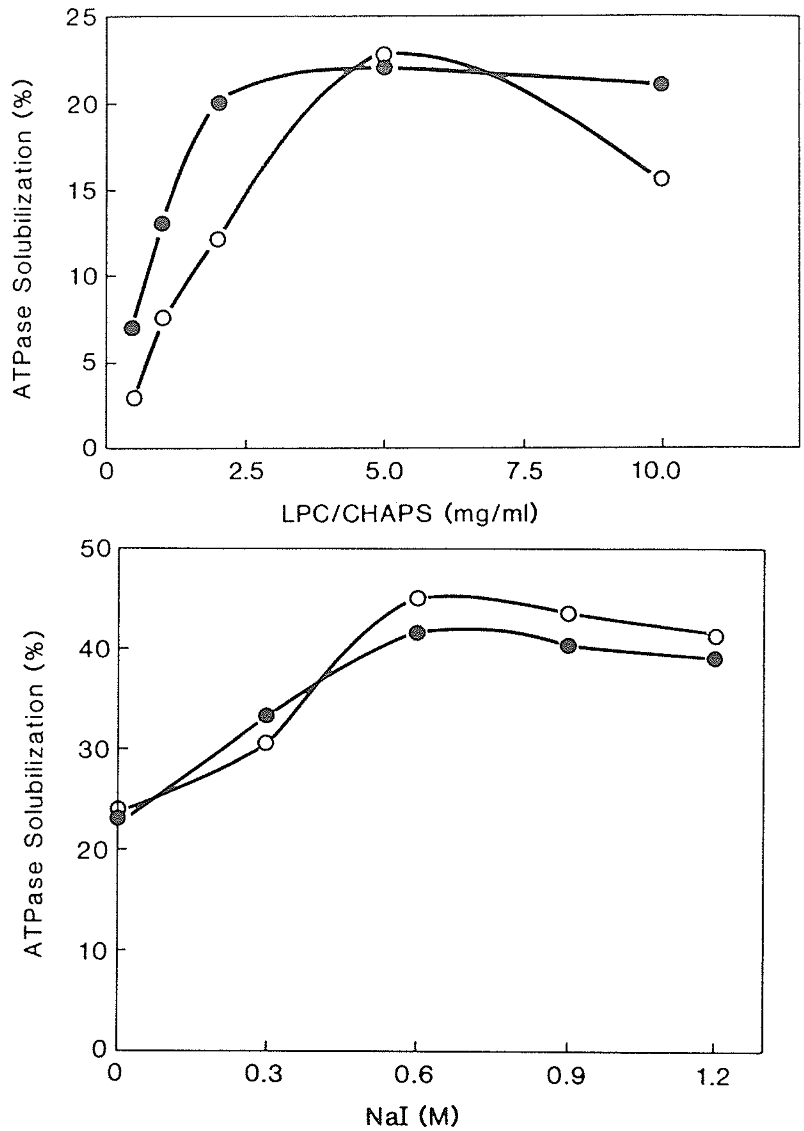

Fig. 4 Upper panel, solubilization of Ca^{2+}/Mg^{2+} ATPase by various concentrations of lysophosphatidylcholine (LPC) and CHAPS. The protein concentration was 10 mg/ml, o Mg^{2+} ATPase activity; ●, Ca^{2+} ATPase activity. Lower panel, effect of NaI on the solubilization of Ca^{2+}/Mg^{2+} ATPase by 5 mg/ml lysophoshatidylcholine and 5 mg/ml CHAPS. The protein concentration was 10 mg/ml. o, Mg^{2+} ATPase activity; ●, Ca^{2+} ATPase activity. Each value is an average of 4 experiments.

1989), had no effects (data not shown). The specific activity of Ca^{2+}/Mg^{2+} ATPase after solubilization was not significantly ($P > 0.05$) different from that in the isolated plasma membranes.

The activity and yield of the enzyme at various steps of purification of the divalent cation dependent ATPase from rat heart plasma membrane are shown in Table 9 whereas the elution profile of the enzyme is given in Fig.5. About 20% of the total Ca^{2+}/Mg^{2+} ATPase activity was recovered from sucrose density gradient centrifugation with about 2 fold increase in specific activity (Fig.5A). The enzyme activity was found at about 20% sucrose concentration (density 1.083). The Ca^{2+}/Mg^{2+} ATPase activity peak was eluted from the Affi-Gel Blue column slightly after the void volume of the column (Fig.5B), indicating that the enzyme was loosely bound to the dye ligand and could be easily washed away. The proteins tightly bound to the column could be eluted with 1M NaCl. About 0.8-1.1% of total Ca^{2+}/Mg^{2+} ATPase activity was recovered from Sepharose 6B column (Fig.5C); the specific activity was 62.1 umol Pi/mg/min for Ca^{2+} ATPase and 80.5 umol Pi/mg/min for Mg^{2+} ATPase. This indicated a 10-fold enrichment of the enzyme activity in purified Ca^{2+}/Mg^{2+} ATPase over the plasma membranes. It is interesting to note that the Ca^{2+} ATPase activity and Mg^{2+} ATPase activity migrated together in sucrose gradient and eluted at the same peak from Affi-Gel Blue column and Sepharose 6B column. Addition of 75 ug/ml phenylmethylsulfonyl fluoride, an inhibitor of proteolysis, in the media at different steps for the preparation of membranes as well as solubilization and purification of Ca^{2+}/Mg^{2+}

Table 9. Yield of the enzyme activities at different steps of purification of Ca^{2+}/Mg^{2+} ATPase from rat heart plasma membrane.

Each value is an average of 6 experiments.

Fraction	Protein (mg)	Specific Activity (umol Pi/mg/min)		Total Activity (umol Pi/min)		Yield of Enzyme Activity (%)	
		Ca^{2+} ATPase	Mg^{2+} ATPase	Ca^{2+} ATase	Mg^{2+} ATPase	Ca^{2+} ATPase	Mg^{2+} ATPase
Plasma membrane	31.3	6.1	8.2	190.9	256.7	100	100
Solubilization	11.8	5.9	7.8	69.6	92.0	36.5	35.8
Sucrose gradient	3.7	10.1	13.4	37.4	49.6	19.6	19.3
Affi-Gel Blue column	0.2	25.3	30.2	5.1	6.0	2.7	2.3
Sepharose 6B column	0.03	62.1	80.5	1.8	2.4	0.9	0.9

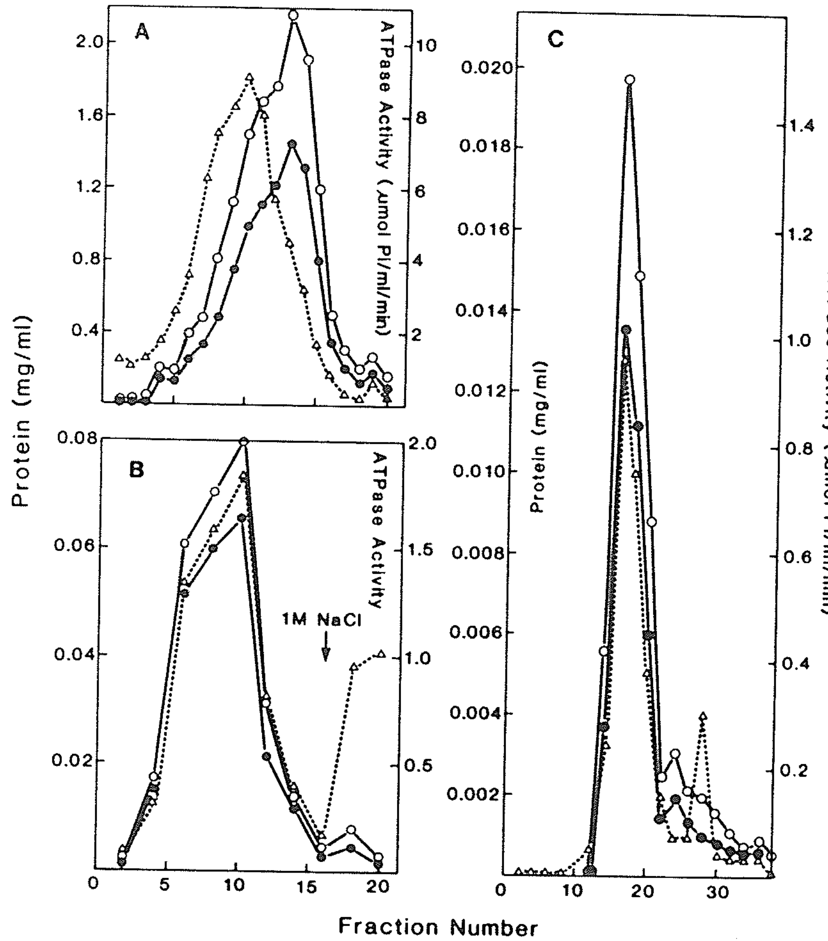

Fig. 5 Elution profile of Ca^{2+}/Mg^{2+} ATPase. A. elution profile of Ca^{2+}/Mg^{2+} ATPase and total protein from sucrose gradient centrifugation. Experimental details are as desccribed under "Experimental Procedures". ●, Ca^{2+} ATPase activity; o, Mg^{2+} ATPase activity; △, protein concentration B. elution profile of Ca^{2+}/Mg^{2+} ATPase pooled from sucrose gradient centrifugation on Affi-Gel Blue column. ●, Ca^{2+} ATPase activity; o, Mg^{2+} ATPase activity; △, protein concentration. C. elution profile of Ca^{2+}/Mg^{2+} ATPase pooled from Affi-Gel Blue column on Sepharose 6B column. ●, Ca^{2+} ATPase activity; o, Mg^{2+} ATPase activity; △, protein concentration.

ATPase did not affect the enzyme activities.

III. Molecular weight and subunit structure of Ca^{2+}/Mg^{2+} ATPase

The electrophoresis of the enzyme in the presence of sodium dedocyl sulfate revealed a major protein band corresponding to molecular weight of 90,000 (Fig.6). This band represented more than 95% of the protein in the gel. The molecular weight of the purified Ca^{2+}/Mg^{2+} ATPase estimated from the elution volumn of the enzyme from calibrated Sepharose 6B column equilibrated with 1% digitonin was about 180,000 (Fig.7). Therefore, the active Ca^{2+}/Mg^{2+} ATPase enzyme appears to be a dimer with two subunits of the same molecular weight.

IV. Enzymatic properties of purified Ca^{2+}/Mg^{2+} ATPase

a. Effect of cations on the enzyme

In one series of experiments, the enzyme was incubated with 4 mM ATP and the hydrolysis measured by employing different concentrations of Ca^{2+} or Mg^{2+} (Fig.8). Lineweaver-Burk analysis of the data indicated a Ka value of 1.47 mM for Ca^{2+} and a Ka value of 2.51 mM for Mg^{2+}. In order to test the ion specificity of the ATPase, the enzyme was incubated with 4 mM ATP and 4 mM of various divalent cations. The ATPase was predominantly activated by Mg^{2+} and Ca^{2+}. Other divalent cations such as Mn^{2+}, Cd^{2+}, Co^{2+}, Sr^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+} were also able to activate the enzyme but to a lesser extent, whereas Ba^{2+} did not activate the Ca^{2+}/Mg^{2+} ATPase (Table 10).

The effects of various divalent cations on the ATPase

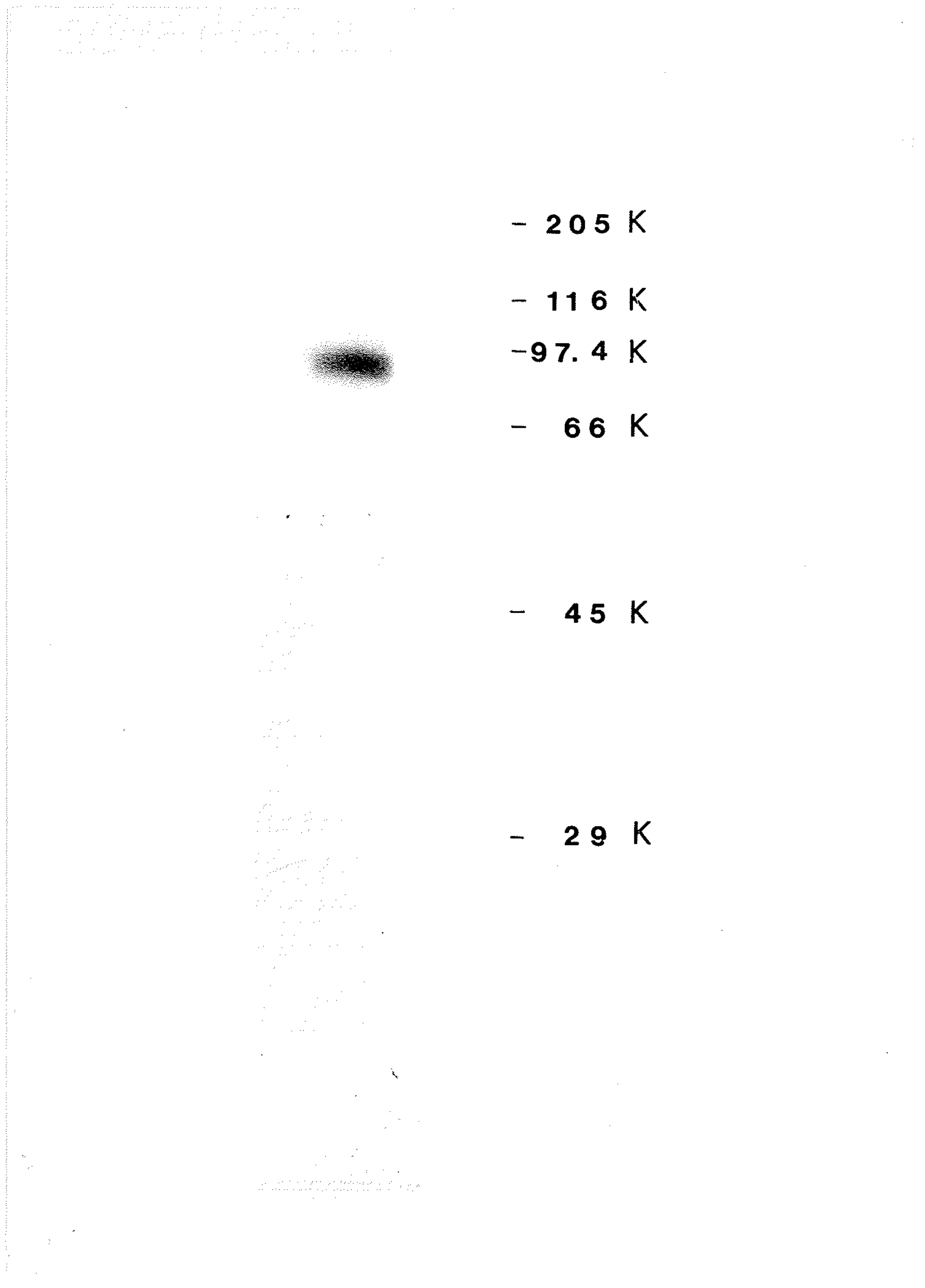

Fig. 6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Ca^{2+}/Mg^{2+} ATPase. One ug of the purified enzyme was applied to 10% polyacrylamide gel. The major protein band corresponds to a molecular weight of about 90,000.

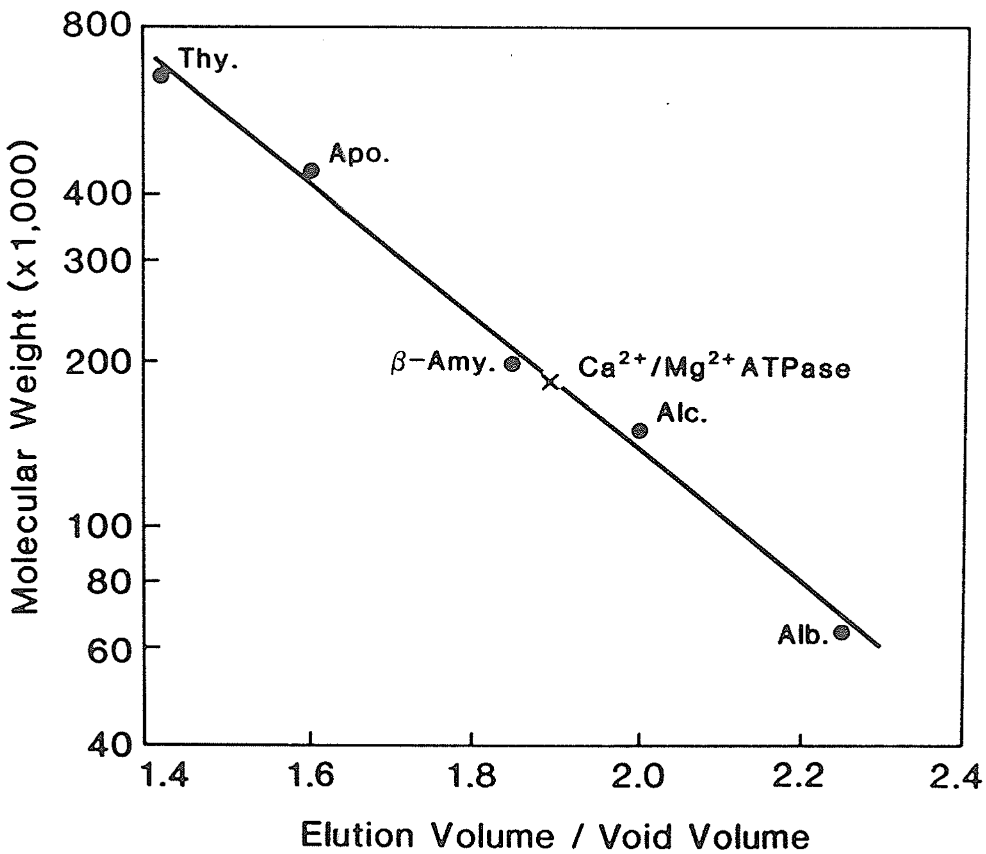

Fig. 7 Gel filtration of Ca^{2+}/Mg^{2+} ATPase on a Sepharose 6B column. The experimental conditions are as described under "Experimental Procedures". The elution volumn of the Ca^{2+}/Mg^{2+} ATPase was compared to those of thyroglobulin (Thy; Mr = 669,000), apoferritin (Apo; Mr = 443,000), β-amylase (β-Amy; Mr = 200,000), alcohol dehydrogenase (Alc; Mr = 150,000), and albumin (Alb; Mr = 66,000).

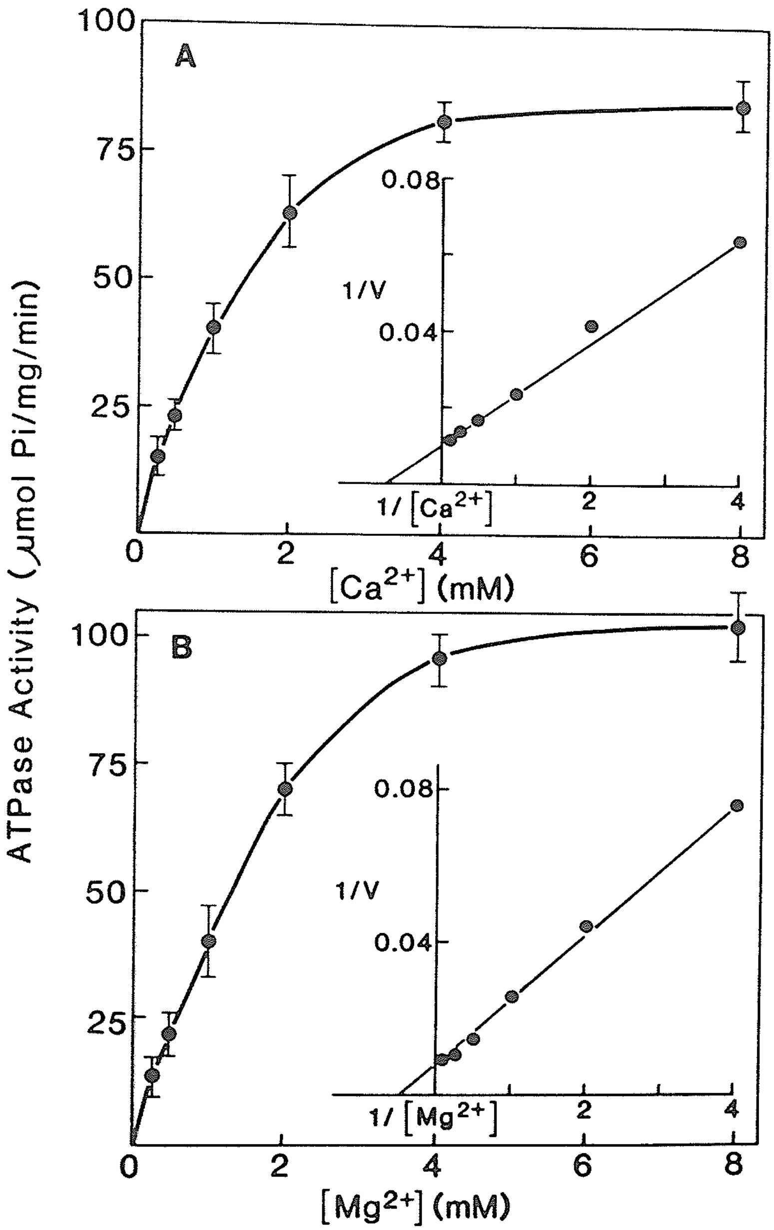

Fig. 8 Effect of different concentrations of Ca^{2+} (A) or Mg^{2+} (B) on the ATP hydrolysis by purified Ca^{2+}/Mg^{2+} ATPase. Each value is mean ± S.E. of 4 different preparations. The inlets are the Lineweaver-Burk plots of the corresponding data.

Table 10. Divalent cation specificity of the purified Ca^{2+}/Mg^{2+} ATPase.

The ATPase activity was assayed in a medium containing 4 mM ATP, 50 mM Tris-HCl, pH 7.4 and 4 mM of various divalent cations for 1 min at 37^{o} C. Each value is a mean ± S.E. of 6 experiments.

Divalent cations	ATPase activity (umol Pi/mg/min)
Mg^{2+}	90.5 ± 10.6
Ca^{2+}	74.4 ± 9.8
Mn^{2+}	51.1 ± 6.1
Cd^{2+}	26.1 ± 3.2
Co^{2+}	25.6 ± 2.9
Sr^{2+}	18.9 ± 2.3
Zn^{2+}	10.0 ± 1.6
Cu^{2+}	6.7 ± 0.9
Ni^{2+}	6.7 ± 0.8
Ba^{2+}	0

activity in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+} are shown in Table 11. The ATPase activity measured in the presence of 4 mM Ca^{2+} plus 4 mM Mg^{2+} was the same as that in the presence of 4-8 mM Mg^{2+}. Since no additive effect of Ca^{2+} plus Mg^{2+} was seen, it seems that these two divalent cations act on the same site of the enzyme. Low concentrations of Ca^{2+} (10 uM) did not stimulate the Mg^{2+} ATPase activity of the enzyme (data not shown). The Ca^{2+}/Mg^{2+} ATPase was inhibited by Cu^{2+}, Zn^{2+}, Ni^{2+} and Cd^{2+} by more than 70% and by Mn^{2+} and Co^{2+} by about 60%. On the other hand, Ba^{2+} and Sr^{2+} did not inhibit these enzyme activities. The monovalent ions had no appreciable effect on the Ca^{2+}/Mg^{2+} ATPase activity (Table 12). No Na^{+}-K^{+}-stimulated ATPase activity was found to be associated with the enzyme because Na^{+} plus K^{+} did not influence its activity. The optimal pH for the Ca^{2+}/Mg^{2+} ATPase activity was around 8.5, although no sharp pH optimum was observed.

b. Substrate of the enzyme

In another series of experiments properties of the purified Ca^{2+}/Mg^{2+} ATPase were studied by varying ATP concentrations in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+} (Fig.9). The reaction was of Michaelis-Menten type. The Lineweaver-Burk analysis of the data indicated that the Km for Ca^{2+} ATPase was 0.34 mM and Vmax was 85.8 umol Pi/mg/min (Fig.9A) whereas the Km for Mg^{2+} ATPase was 0.48 mM with a Vmax of 111.4 umol Pi/mg/min (Fig.9B) as shown in Table 13, the Ca^{2+}/Mg^{2+} ATPase was selective for nucleoside 5'-triphosphates. The enzyme hydrolyzed

Table 11. Effect of different divalent cations on purified Ca^{2+}/Mg^{2+} ATPase of rat heart plasma membrane.

The purified Ca^{2+}/Mg^{2+} ATPase was incubated in the presence of 4 mM ATP, 4mM $CaCl_2$ or 4 mM $MgCl_2$ and 4 mM of divalent cations. The ATPase activities are expressed as percentages of Ca^{2+}ATPase or Mg^{2+} ATPase in the absence of any other divalent cations. Each value is a mean ± S.E. of 4 experiments.

Ions added	Relative Ca^{2+} ATPase activity	Relative Mg^{2+} ATPase activity
None	100	100
Mg^{2+}	126 ± 10	109 ± 12
Ba^{2+}	98 ± 4	104 ± 6
Sr^{2+}	92 ± 4	89 ± 7
Mn^{2+}	42 ± 2	40 ± 2
Co^{2+}	43 ± 2	41 ± 3
Cd^{2+}	31 ± 3	26 ± 2
Ni^{2+}	23 ± 1	27 ± 1
Zn^{2+}	9 ± 2	12 ± 3
Cu^{2+}	5 ± 1	15 ± 2

Table 12. Effect of monovalent ions on the purified Ca^{2+}/Mg^{2+} ATPase activity.

The ATPase activity was assayed in a medium containing 4 mM ATP, 50 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$ or 4 mM $CaCl_2$ and various ions as indicated for 1 min at 37°C. Each value is a mean ± S.E. of 4 experiments.

Ions added	Concentration (mM)	Ca^{2+} ATPase (umol Pi/mg/min)	Mg^{2+} ATPase (umol Pi/mg/min)
None		73.7 ± 8.6	90.5 ± 10.1
Na^+	100	73.3 ± 8.2	82.8 ± 9.1
K^+	100	73.9 ± 9.3	85.0 ± 8.9
$Na^+ + K^+$	100 + 20	74.5 ± 7.8	81.5 ± 8.5
HCO_3^-	50	77.8 ± 8.4	92.8 ± 9.8
pH 6.0		67.6 ± 7.4	84.5 ± 7.6
pH 8.5		77.4 ± 8.1	102.0 ± 11.3
pH 9.5		50.0 ± 5.8	74.2 ± 6.5

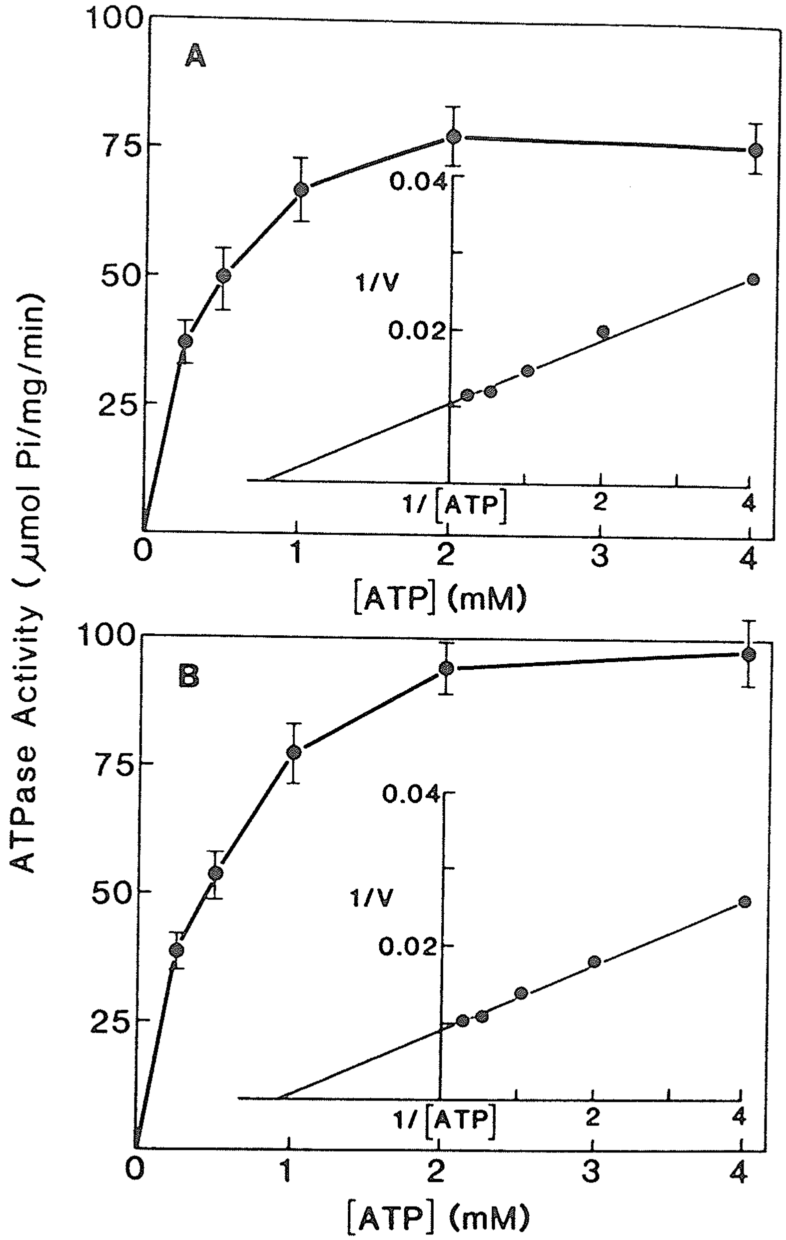

Fig. 9 Effect of different concentrations of ATP on the activities of purified Ca^{2+} ATPase (A) and Mg^{2+} ATPase (B). Each value is a mean ± S.E. of 4 different preparations. The inlets are the Lineweaver-Burk plots of the corresponding data.

Table 13. Substrate specificity of the purified Ca^{2+}/Mg^{2+} ATPase.

The purified Ca^{2+}/Mg^{2+} ATPase was incubated in the assay medium containing 50 mM Tris-HCl, pH 7.4, 4 mM of Ca^{2+} or 4 mM Mg^{2+} and 4 mM of nucleotides. pNNP: p-nitrophenyl phosphate. Each value is a mean ± S.E. of 4 experiments.

	Hydrolysis rate (umol Pi/mg/min)	
Substrate	in the presence of Ca^{2+}	in the presence of Mg^{2+}
ATP	78.7 ± 5.8	113.0 ± 9.6
CTP	48.6 ± 5.1	40.0 ± 4.6
ITP	28.9 ± 4.7	63.8 ± 8.4
UTP	66.7 ± 5.5	72.9 ± 5.5
GTP	57.4 ± 5.2	99.0 ± 6.7
ADP	0	0
pNPP	0	0

GTP, UTP and CTP in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+}, although at a somewhat lower rate than it hydrolyzed ATP. On the other hand, ADP and p-nitrophenyl phosphate were not hydrolyzed by the enzyme.

c. Effect of inhibitors

Table 14 shows the effects of various inhibitors on the purfied Ca^{2+}/Mg^{2+} ATPase activity. The Ca^{2+}/Mg^{2+} ATPase activity was insensitive to the mitochondrial ATPase inhibitors oligomycin and N,N'-dicyclohexylcarbodiimide, but sodium azide at high concentration inhibited the enzyme slightly. Verapamil, which is a well known Ca^{2+} channel blocker, was without effect on the ATPase activity. Vanadate, which inhibits Na^{+} -K^{+} ATPase and Ca^{2+}-stimulated ATPase of sarcolemma, did not inhibit the Ca^{2+}/Mg^{2+} ATPase activity at high concentration. The enzyme was also not sensitive to ouabain, a well known inhibitor of the Na^{+}-K^{+} ATPase. On the other hand, gramicidin S, an antibiotic which was found to be a potent inhibitor of divalent cation ATPases of heart sacolemma (Zhao and Dhalla, 1989), also inhibited the purified Ca^{2+}/Mg^{2+} ATPase. The IC_{50} for the inhibition of the Ca^{2+}/Mg^{2+} ATPase by gramacidin S varied between 10 to 20 uM. The calmodulin antagonist trifluoperazine also inhibited the Ca^{2+}/Mg^{2+} ATPase activity at high concentrations, whereas this agent showed negligible effect at low concentrations, which are sufficient to inhibit calmodulin.

Table 14. Effect of some inhibitors on the purified Ca^{2+}/Mg^{2+} ATPase activity.

The ATPase activity was assayed in a medium containing 4 mM ATP, 50 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$ or 4 mM $CaCl_2$ and various inhibitors as indicated. Each value is a mean ± S.E. of 4 experiments.

DCCD: N,N'-dicyclohexylcarbodiimide. NEM: N-ethylmaleimide.

Inhibitor	Concentration	Ca^{2+} ATPase (umol Pi/mg/min)	Mg^{2+} ATPase (umol Pi/mg/min)
None		58.3 ± 6.3	73.8 ± 8.6
Oligomycin	50 uM	58.3 ± 7.1	72.6 ± 8.0
NaN_3	5 mM	68.8 ± 5.1	65.5 ± 7.4
DCCD	50 uM	53.6 ± 6.2	67.8 ± 7.5
Verapamil	10 uM	57.4 ± 5.2	74.2 ± 7.9
Vanadate	1 mM	47.6 ± 5.1	70.2 ± 7.8
Ouabain	1 mM	58.4 ± 4.2	72.6 ± 5.8
Gramicidin S	5 uM	41.7 ± 5.1	64.2 ± 7.2
	20 uM	13.1 ± 2.0	36.9 ± 4.3
Trifluoperazine	10 uM	54.6 ± 6.4	70.2 ± 7.9
	50 uM	15.5 ± 1.9	39.3 ± 4.1

V. Chemical composition and phospholipid requirements for purified Ca^{2+}/Mg^{2+} ATPase

a. Amino acid composition and peptide mapping

As indicated earlier, the Ca^{2+}/Mg^{2+} ATPase has two subunits with a molecular weight of about 90,000 each. The amino acid composition of the heart Ca^{2+}/Mg^{2+} ATPase in Table 15 reveal that glutamic acid, leucine and serine are major constituents whereas histidine, cysteine and methionine were minor components. Trytophan was not detectable. Cyanogen bromide peptide map (Fig. 10) showed that the 90,000 peptide was cleaved into three fragments with molecular weights of about 15,000, 45,000 and 55,000. The fact that the heart Ca^{2+}/Mg^{2+} ATPase is more resistant to CNBr cleavage than either sarcoplasmic reticular Ca^{2+}-stimulated ATPase or transverse tubular Mg^{2+} ATPase (Brandl et al, 1986; Kirley, 1988) is consistant with the finding that this enzyme has less methionine per peptide.

b. Phospholipid requirement for the Ca^{2+}/Mg^{2+} ATPase activity

The purified Ca^{2+}/Mg^{2+} ATPase was found to be associated with a large amount of phospholipids (Table 16). The phospholipid to protein ratio in the purified enzyme preparation was about 60 times higher than that in plasma membrane. It is possible that the enzyme was re-incorporated back into the lipid environment during the purification procedure. Higher levels of lysophosphatidylcholine in the purified Ca^{2+}/Mg^{2+} ATPase in comparison to plasma membrane may be due to the fact that this

Table 15. Amino acid composition of the purified Ca^{2+}/Mg^{2+} ATPase.

The values were calculated assumuing a molecular mass of 180 K Da. Values for cysteine also include that for cystine.

Amino Acid	Residues/protein molecule	Relative amount (mole %)
Aspartic acid	153	9.3
Threonine	89	5.4
Serine	163	9.9
Glutamic acid	174	10.5
Proline	74	4.5
Glycine	142	8.6
Alanine	126	7.6
Valine	113	6.8
Methionine	10	0.6
Isoleucine	93	5.6
Leucine	171	10.3
Tyrosine	49	3.0
Phenylalanine	76	4.6
Histidine	32	2.0
Lysine	85	5.1
Arginine	26	4.6
Cysteine	33	2.0
Tryptophan	0	0

Table 16. Phospholipid and cholesterol composition of rat heart plasma membrane and purified Ca^{2+}/Mg^{2+} ATPase preparation.

The individual phospholipid content is expressed as percentage of total phospholipids. PC: phosphatidycholine; PE: phosphatidylethanolamine.

	Membrane	Purified Ca^{2+}/Mg^{2+} ATPase
Phosphatidylcholine (%)	43.6	27.8
Phosphatidylethanolamine (%)	28.1	20.8
Phosphatidylserine (%)	2.8	16.2
Phosphatidylinositol (%)	7.1	3.2
Sphingoinyelin (%)	8.3	7.1
Lysophosphatidylcholine (%)	3.0	17.0
Diphosphatidylglycerol (%)	4.5	2.1
Unknown (%)	2.6	5.8
Total phospholipid phosphorus (umol Pi/mg protein)	0.37	19.7
PC:PE ratio	1.55	1.34
Cholesterol content (umol/mg)	0.094	0.75
Cholesterol:phospholipid ratio	0.25	0.04

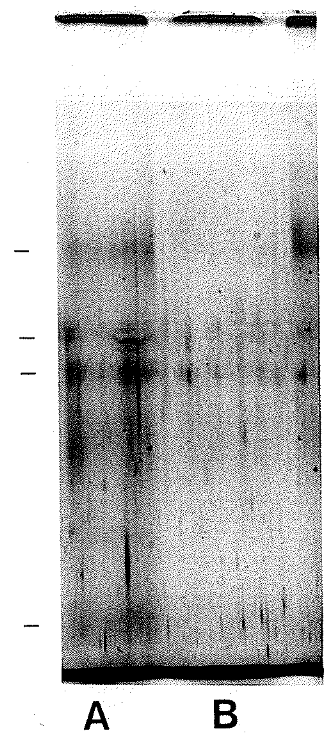

Fig. 10 Cyanogen bromide peptide map of purified Ca^{2+}/Mg^{2+} ATPase. The CNBr treated protein was run on a 12% sodium dodecyl sulfate gel and silver stained. The protein was treated with 100 (A) and 1000 (B) molar excess cyanogen bromide. The first peptide band from the top is the original 90,000 peptide of Ca^{2+}/Mg^{2+} ATPase, while the other three bands have molecular weights of 55,000, 45,000, and 15,000, respectively.

lipid was used for the solubilization of the enzyme. However, it is interesting to note that phosphatidylserine was enriched with the purified Ca^{2+}/Mg^{2+} ATPase, while other phospholipids showed correspondent decrease in their relative contents. Cholesterol was also enriched in the purified Ca^{2+}/Mg^{2+} ATPase preparation but not as much as the phospholipids and thus the ratio of cholesterol to phospholipid was decreased.

From the data given in Table 17, it seems that the phospholipids associated with the purified protein play some role in the maintenance of Ca^{2+}/Mg^{2+} ATPase activity. Phospholipases A and C inhibited the membrane-bound Ca^{2+}/Mg^{2+} ATPase, which is consistant with earlier reports (Anand-Srivastava and Dhalla, 1987b; Zhao et al., 1989). Phospholipase A and C were also found to inhibit the purified Ca^{2+}/Mg^{2+} ATPase by about 50%. This inhibition was slightly lower than that in plasma membrane, probably due to a higher amount of the lipids associated with the enzyme.

c. Polysaccharide content and concanavalin A interaction

The purified Ca^{2+}/Mg^{2+} ATPase was found to contain 80 ug/mg protein polysaccharide, which was about 8 times higher than that found in the plasma membrane, whereas sialic acid content of the purified enzyme was enriched about 4 times (Table 18). The results in Table 18 indicate that neuraminidase decreased polysaccharide content by 50%. Membrane bound Ca^{2+}/Mg^{2+} ATPase activity was inhibited by neuraminidase by about 50 - 60%, whereas the purified Ca^{2+}/Mg^{2+} ATPase was inhibited by about

Table 17. Effect of phospholipase A, phospholipase C and phospholipase D on plasma membrane-bound and purified Ca^{2+}/Mg^{2+} ATPase activities.

* Significantly different from control (P<0.05). Each value is a mean ± S.E. of 4 experiments.

	ATPase activity (umol Pi/mg/min)			
	Ca^{2+} ATPase		Mg^{2+} ATPase	
	membrane-bound	purified	membrane-bound	purified
Control	6.8 ± 0.5	88.8 ± 9.2	8.4 ± 0.6	104.2 ± 9.4
Phospholipase A	1.9 ± 0.2*	40.6 ± 3.6*	2.3 ± 0.2*	42.3 ± 4.8*
Phospholipase C	2.9 ± 0.2*	41.5 ± 3.1*	2.4 ± 0.2*	45.7 ± 5.7*
Phospholipase D	6.2 ± 0.6	85.3 ± 4.2	8.0 ± 0.7	97.2 ± 5.6

Table 18. Effect of neuraminidase on rat heart plasma membrane-bound and purified Ca^{2+}/Mg^{2+} ATPase activities and polysaccharide content.

* Significantly different from control ($P<0.05$). Each value is a mean ± S.E. of 4 experiments. N.D.: not determined.

	Membrane-bound ATPase		Purified ATPase	
	Control	Treated	Control	Treated
Polysaccharide (ug/mg protein)	10.4 ± 0.8	5.2 ± 0.3*	80.1 ± 7.7	40.8 ± 4.1*
Sialic acid (nmol/mg protein)	23.9 ± 1.6	N.D.	107 ± 23	N.D.
Ca^{2+} ATPase (umol Pi/mg/min)	7.33 ± 0.71	3.42 ± 0.44*	62.1 ± 5.8	40.9 ± 3.4*
Mg^{2+} ATPase (umol Pi/mg/min)	9.18 ± 0.88	3.78 ± 0.37*	85.6 ± 6.2	55.2 ± 5.4*

30 - 40%. The periodic acid-Schiff staining of sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified Ca^{2+}/Mg^{2+} ATPase showed that the enzyme was stained positively (Fig.11), providing further evidence that the Ca^{2+}/Mg^{2+} ATPase is a glycoprotein.

As in the case of membrane bound Ca^{2+}/Mg^{2+} ATPase, the purified Ca^{2+}/Mg^{2+} ATPase also showed ATP-dependent inactivation (Fig.12). The time course of the enzyme reaction was not linear with time (Fig.12 A and C) and in fact the rate of ATP hydrolysis by the Ca^{2+}/Mg^{2+} ATPase decayed exponentially (Fig.12 B and D). The first-order rate constant of inactivation was 0.18 min^{-1} for Ca^{2+} ATPase and 0.23 min^{-1} for Mg^{2+} ATPase at 37°C. Addition of 1 mM KH_2PO_4, ADP, and AMP in the assay medium did not show significant change in the initial rates of ATPase activities. This indicated that the nonlinearity of the reaction was not due to the product inhibition. On the other hand, concanavalin A, which is known to interact with polysaccharides, partially prevented the ATP-dependent inactivation (Fig.12). The initial rate of ATP hydrolysis by purified Ca^{2+}/Mg^{2+} ATPase was not changed by concanavalin A significantly, but the inactivation rate constant was decreased by concanavalin A to 0.097 min^{-1} and 0.15 min^{-1} for Ca^{2+} ATPase and Mg^{2+} ATPase activity, respectively. Concanavalin A was also found to bind to the purified Ca^{2+}/Mg^{2+} ATPase in a concentration dependent and saturable manner (Fig.13). Scatchard analysis of the data showed a straight line with a K_D of 576 nM and a Bmax of 4.5 nmol/mg protein. The mole to mole

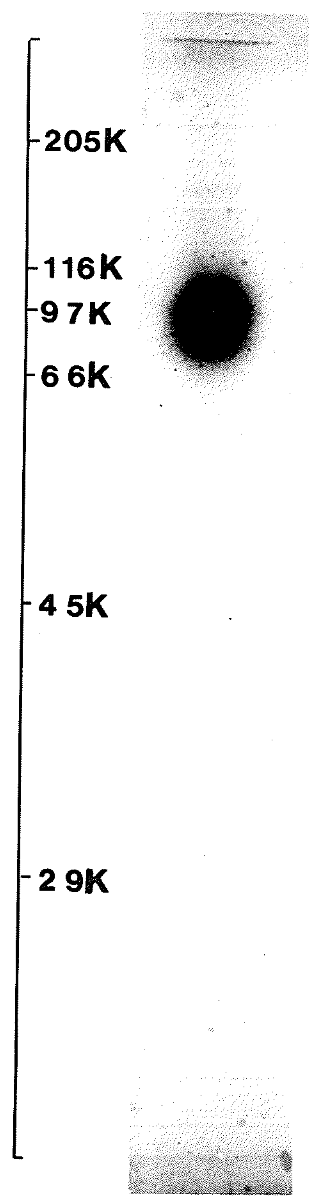

Fig. 11 Periodic acid-Schiff staining of the purified Ca^{2+}/Mg^{2+} ATPase in sodium dodecyl sulfate electrophoresis gel. The positively stained protein band has a molecular weight of about 90,000.

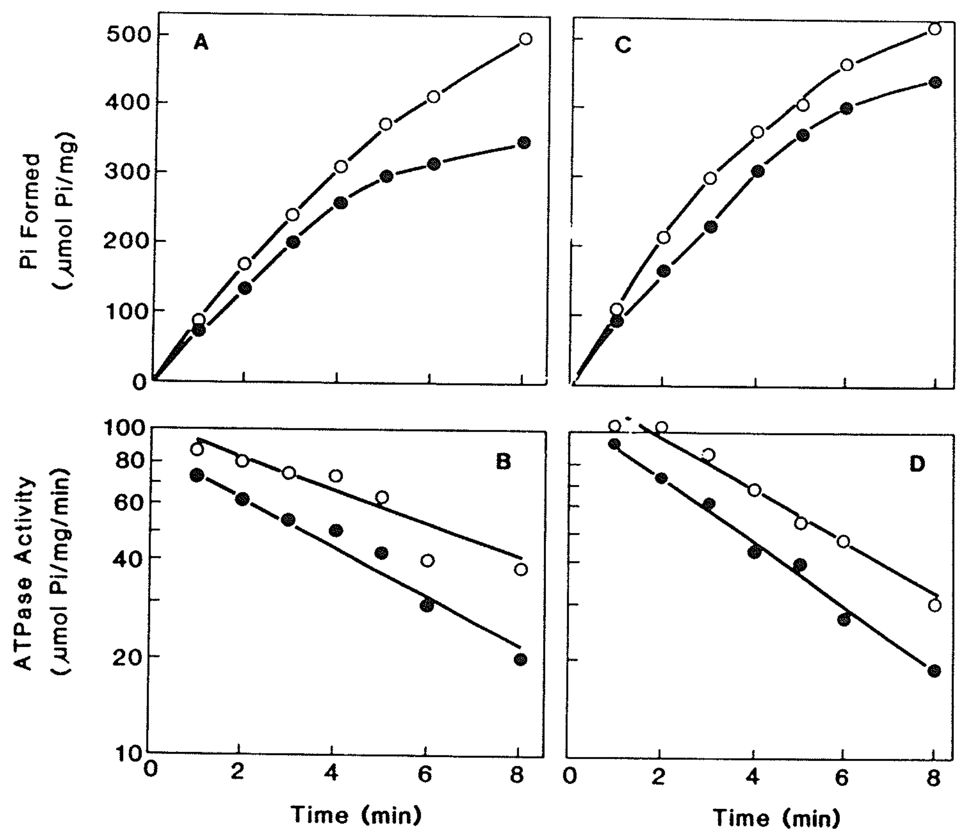

Fig. 12 Effect of concanavalin A (10 ug/ml) on time course of ATP hydrolysis by purified Ca^{2+}/Mg^{2+} ATPase. A and B, Ca^{2+}-ATPase; C and D, Mg^{2+} ATPase activity. A and C, time course of ATP hydrolysis by purified Ca^{2+}/Mg^{2+} ATPase in the presence (o) or absence (●) of concanavalin A. B and D, changes of purified Ca^{2+}/Mg^{2+} ATPase activities with time in the presence (o) or absence (●) of concanavalin A.

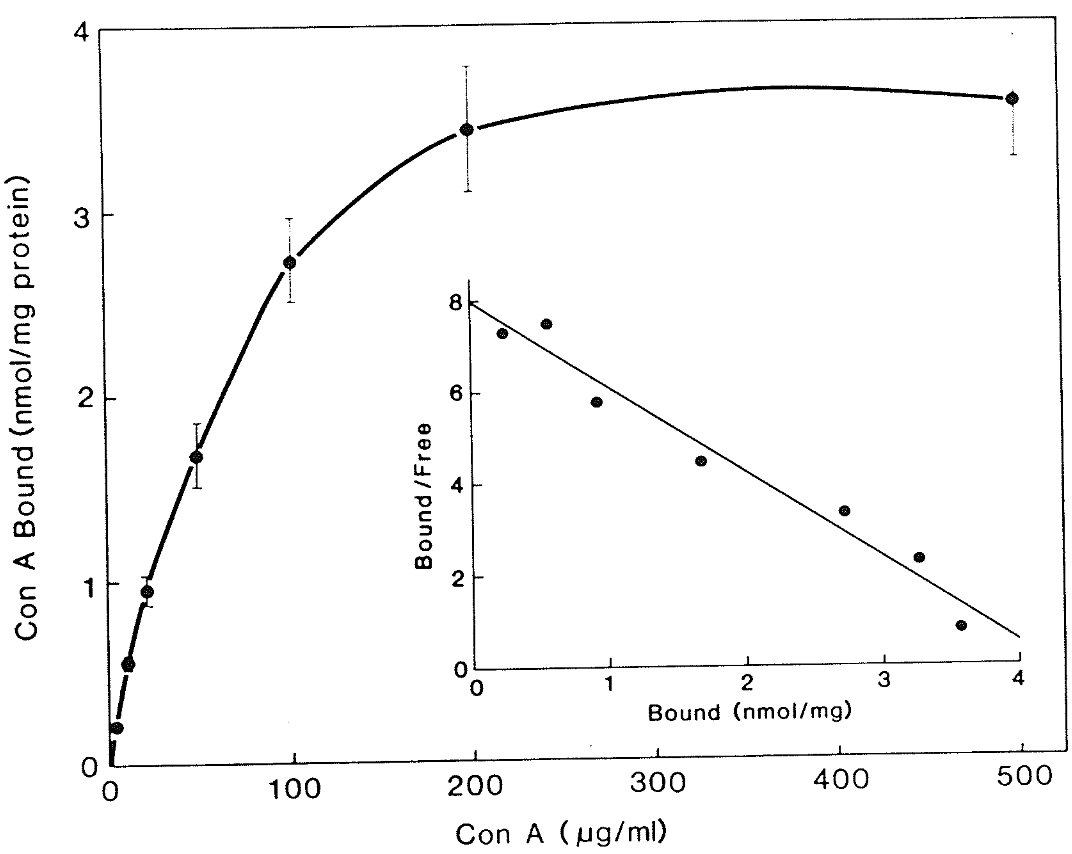

Fig. 13 Concanavalin A binding to the purified Ca^{2+}/Mg^{2+} ATPase. Each value is a mean ± S.E. of 4 experiments. The inlet is the Scatchard plot of the same data.

stoichiometry of concanavalin A to purified Ca^{2+}/Mg^{2+} ATPase enzyme (Mr = 180,000) was calculated at 0.8:1. The affinity of the enzyme to concanavalin A was similar to that for the plasma membrane of rat heart (Zhao et al., 1989).

VI. Ligand binding with purified Ca^{2+}/Mg^{2+} ATPase

a. ATPrS binding with Ca^{2+}/Mg^{2+} ATPase

ATPrS was found to bind to the purified Ca^{2+}/Mg^{2+} ATPase specifically (Fig.14). The binding was saturable at 10 uM concentration of ATPrS. Scatchard analysis showed two apparent affinity sites with K_D of 50.9 $\pm$ 0.8 nM for high affinity site and 1160 $\pm$ 198 nM for low affinity site; the Bmax values were 8.71 $\pm$ 0.16 nmol/mg protein and 145 $\pm$ 9.7 nmol/mg protein, respectively. The mole to mole ratio of maximal high affinity ATPase binding to Ca^{2+}/Mg^{2+} ATPase subunit (Mr = 90,000) was 0.8, indicating that there was one high affinity ATPrS binding site on each Ca^{2+}/Mg^{2+} ATPase subunit. Phospholipases A, C and D as well as neuraminidase had no effect on th ATPrS binding(Table 19); this indicates that the ATPrS binding is due to the enzyme protein per se.

b. Ca^{2+} binding with Ca^{2+}/Mg^{2+} ATPase

Fig.15 shows that Ca^{2+} bound to the purified Ca^{2+}/Mg^{2+} ATPase in a concentration-dependent and saturable manner. Scatchard plot analysis of the data showed a straight line with a K_D of 0.384 mM and a Bmax of 1.85 umol/mg protein. The K_D value of Ca^{2+} binding to the purified enzyme corresponded to that of low affinity Ca^{2+} binding site of sarcolemma (Gupta et al, 1989), whereas no high affinity Ca^{2+} binding site was found

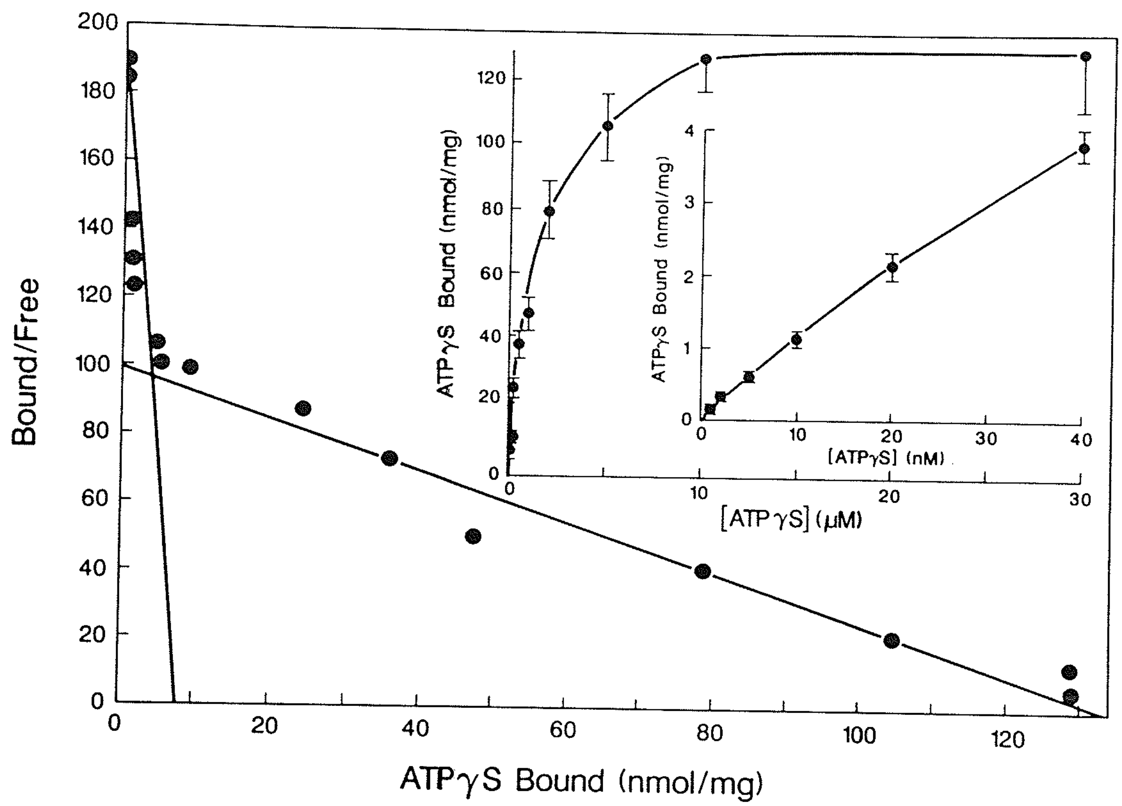

Fig. 14 Scatchard analysis of ATPrS binding to purified Ca^{2+}/Mg^{2+} ATPase. Inlet, ATPrS binding at various ATPrS concentrations.

Table 19. Effects of phospholipases A, C, D and neuraminidase treatment on the on the ATPrS binding and Ca^{2+} binding to purified Ca^{2+}/Mg^{2+} ATPase.

* Significantly different from control ($P<0.05$). Each value is a mean ± S.E. of 4 experiments.

Treatment	(% of control) ATPrS bound		Ca^{2+} bound	
	10 nM	20 uM	50 uM	1.25 mM
Phospholipase A	92 ± 4.2	89 ± 5.7	70.7 ± 1.4*	77.8 ± 4.1*
Phospholipase C	99 ± 3.6	102 ± 2.4	97.0 ± 7.8	98.5 ± 5.6
Phospholipase D	101 ± 2.4	105 ± 3.6	103.6 ± 1.6	109.1 ± 2.1
Neuraminidase	110 ± 3.9	100 ± 3.9	81.3 ± 5.6*	76.6 ± 7.2*

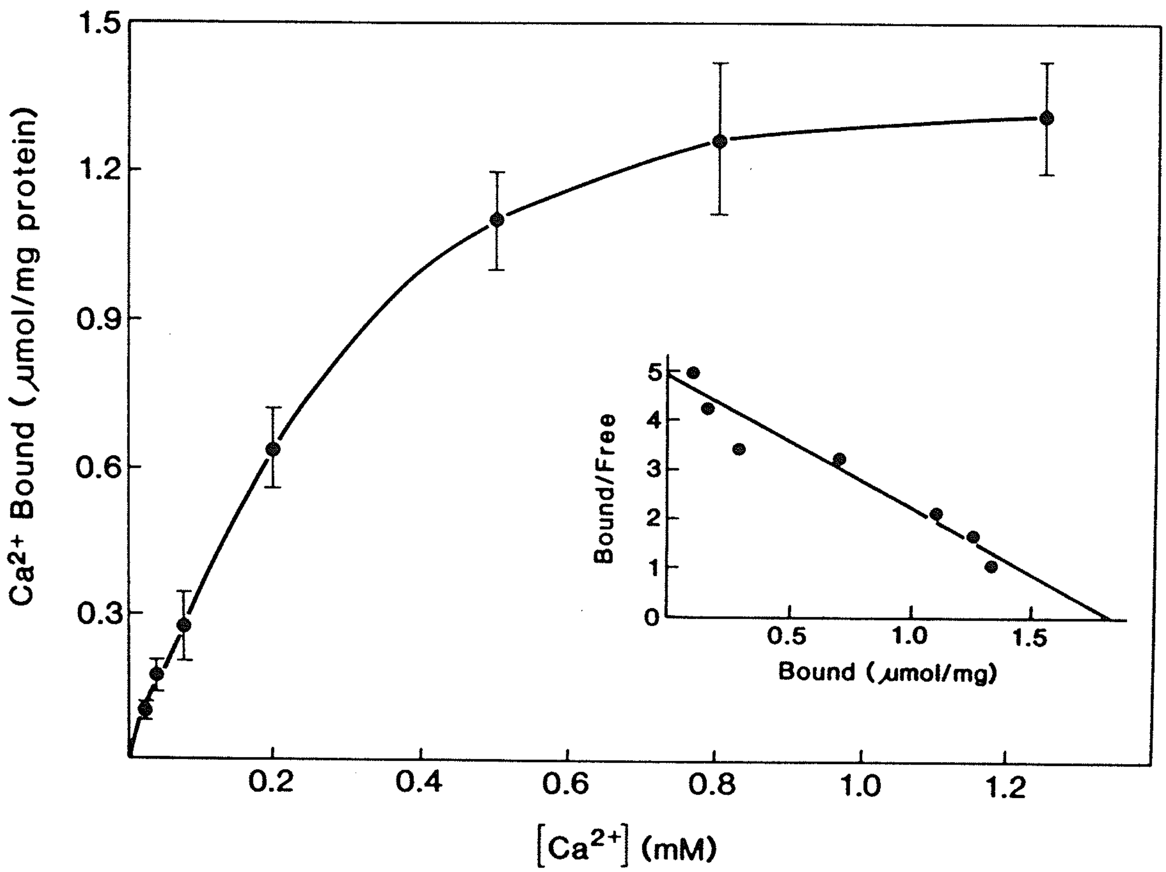

Fig. 15 Ca^{2+} binding to the purified Ca^{2+}/Mg^{2+} ATPase. Each value is a mean ± S.E. of 4 experiments. The inlet is the Scatchard plot of the same data.

in the purified Ca^{2+}/Mg^{2+} ATPase. The maximal Ca^{2+} binding per mg protein to the purified Ca^{2+}/Mg^{2+} ATPase was 10 times higher than the maximal Ca^{2+} binding to rat heart sarcoemmal membrane. This figure is consistant with the purification factor of the enzyme activity. However, if we take 180,000 as the molecular weight of Ca^{2+}/Mg^{2+} ATPase, the stoichiometry of Ca^{2+} binding to the protein would be about 300 to 1. The results in Table 19 show that phospholipase A and neuraminidase significantly reduced the Ca^{2+} binding to the enzyme, whereas phospholipase D had no effect. This indicated that Ca^{2+} is not only bound to the enzyme protein but also to the phospholipids and polysaccharrides associated with the Ca^{2+}/Mg^{2+} ATPase.

ATP inhibited the Ca^{2+} binding to the enzyme, perhaps due to its chelating effect for Ca^{2+} (Table 20). Mg^{2+} did not affect the Ca^{2+} binding significantly, whereas Ni^{2+}, Mn^{2+}, Zn^{2+} at 1 mM concentration significantly inhibited the Ca^{2+} binding (Table 20). Verapamil, a Ca^{2+} channel antagonist, had no effect on Ca^{2+}-binding to the enzyme. The Ca^{2+}-pump activity (ATP and Mg^{2+}-dependent Ca^{2+} uptake of the sarcolemmal membrane) was not detectable with the purified Ca^{2+}/Mg^{2+} ATPase. In order to show the specificity of Ca^{2+} binding with purified Ca^{2+}/Mg^{2+} ATPase we also measured [^{3}H] prazosin (0.1 to 10 nM), [^{3}H] dihydroalprenolol (0.1 to 10 nM), and Ca^{2+} channel blocker [^{3}H]PN200-110 (0.1 to 1000 nM) binding to the purified enzyme preparation, but no binding activity for these ligands was observed.

Table 20. Effect of some agents on Ca^{2+}-binding to purified Ca^{2+}/Mg^{2+} ATPase.

Control values for Ca^{2+} binding at 50 uM and 1.25 mM concentrations were 0.25 umol/mg and 9.9 umol/mg, respectively. * Significantly different from control value. Each value was a mean+ S.E. of 4 experiments.

Agents		Ca^{2+} binding (% of control)	
		50 uM Ca^{2+}	1.25 mM Ca^{2+}
ATP	0.1 mM	106 $\pm$ 15.3	94.2 $\pm$ 15.2
	1.0 mM	4.6 $\pm$ 2.7*	32.9 $\pm$ 4.5*
$MgCl_2$	0.1 mM	128 $\pm$ 11.6	109 $\pm$ 13.9
	1.0 mM	81.4 $\pm$ 9.9	75.6 $\pm$ 9.9
$NiCl_2$	0.1 mM	62.6 $\pm$ 7.7*	94.5 $\pm$ 11.9
	1.0 mM	33.3 $\pm$ 4.7*	51.3 $\pm$ 5.2*
$MnCl_2$	0.1 mM	74.2 $\pm$ 10.1	106 $\pm$ 11.1
	1.0 mM	37.5 $\pm$ 6.2*	37.4 $\pm$ 6.3*
$ZnCl_2$	0.1 mM	61.7 $\pm$ 7.3*	84.7 $\pm$ 7.9
	1.0 mM	24.6 $\pm$ 5.6*	23.7 $\pm$ 4.2*
Verapamil	10 uM	95.7 $\pm$ 13.4	109 $\pm$ 9.6
	100 uM	84.2 $\pm$ 14.1	105 $\pm$ 13.4

E. DISCUSSION

The Ca^{2+}/Mg^{2+} ATPase purified in this study was found to exhibit biochemical properties similar to the membrane-bound enzyme in the heart plasma membrane. For example, both preparations were selective for nucleotide 5'-triphosphate and were activated by millimolar concentrations of various divalent cations, with Mg^{2+} and Ca^{2+} being most potent. The kinetic properties for both preparations as well as pH optima of both activities were comparable. Furthermore, the inhibitors which decreased the membrane-bound Ca^{2+}/Mg^{2+} ATPase were also observed to inhibit the purified enzyme. Thus it appears that the purified Ca^{2+}/Mg^{2+} ATPase assumes the native configuration as is in the plasma membrane. In fact the purified Ca^{2+}/Mg^{2+} ATPase from plasma membrane seems to be a new enzyme which can be easily distinguished from other ATPases in the heart cell. In this regard, it may be noted that the Ca^{2+}/Mg^{2+} ATPase purified from plasma membrane was activated fully by 4-8 mM Ca^{2+} or Mg^{2+}, whereas Ca^{2+}-stimulated ATPases (Ca^{2+} pump ATPases) from sarcolemma and sarcoplasmic reticulum are fully activated by 5-10 uM Ca^{2+} and concentrations of Ca^{2+} higher than 100 uM are inhibitory (Tada et al., 1978). In contrast to the Ca^{2+}-stimulated ATPases, the Ca^{2+}/Mg^{2+} ATPase was not inhibited by vanadate and it did not require the presence of both Ca^{2+} and Mg^{2+} for its activity. Furthermore, the purified Ca^{2+}/Mg^{2+} ATPase, unlike the Ca^{2+}-stimulated ATPase from the sarcolemma and sarcoplasmic reticulum, was not activated by micromolar concentrations of

Ca^{2+} in the presence of Mg ATP. The lack of response of Ca^{2+}/Mg^{2+} ATPase to some mitochondrial inhibitors such as oligomycin and NaN_3 distinguishes this enzyme from the mitochondrial ATPase. Furthermore, HCO_3^- which stimulates the mitochondrial ATPase failed to affect the plasma membrane Ca^{2+}/Mg^{2+} ATPase activity. Similarly, the purified Ca^{2+}/Mg^{2+} ATPase was insensitive to the inhibitors of the Na^+-K^+ ATPase such as ouabain and vanadate and was not stimulated by Na^+ plus K^+. The purified Ca^{2+}/Mg^{2+} ATPase also differs from the myofibrillar ATPase since Ca^{2+}/Mg^{2+} ATPase was shown to be tightly associated with the membrane (Zhao et al., 1989); it was only solubilized by the use of detergents whereas myosin can be extracted with high salt solutions. In addition, the myofibrillar ATPase has little activity in the presence of Mg^{2+} alone whereas Ca^{2+}/Mg^{2+} ATPase showed a higher activity with Mg^{2+} than with Ca^{2+}. The molecular weight and subunit structure of the purified Ca^{2+}/Mg^{2+} ATPase also distinguishes it from other ATPases in the heart cell.

Several attempts have been made to purify the low affinity Ca^{2+}/Mg^{2+} ATPase from cell membrane. Trypsin treatment of the rat heart heavy sarcolemmal membrane solubilized a Ca^{2+}-dependent ATPase with a molecular weight of 67,000 (Tuana and Dhalla, 1982); however, it should be pointed out that unlike the Ca^{2+}/Mg^{2+} ATPase, the Ca^{2+}-dependent ATPase showed a negligible activity in the presence of Mg^{2+}. The Ca^{2+}-dependent ATPase may exist in a hydrophillic environment because the enzyme was solubilized without the use of detergents, while the Ca^{2+}/Mg^{2+} ATPase appears to be an intrinsic membrane protein situated in the hydrophobic lipid environment. Furthermore, unlike the Ca^{2+}/Mg^{2+} ATPase, the Ca^{2+}-dependent ATPase exhibited Ca^{2+}-Na^{+} antagonistsic sites and was markedly inhibited by Mg^{2+} (Tuana and Dhalla, 1982). It has also been shown that the Ca^{2+}-dependent ATPase was an ecto-enzyme of the heart cell membrane since unlike the Ca^{2+}/Mg^{2+} ATPase, it was solubilized from cardiac myocytes with trypsin treatment. While all these characteristics of the Ca^{2+}-dependent ATPase suggest that it may be a distinctly different enzyme from the Ca^{2+}/Mg^{2+} ATPase, the Ca^{2+}/Mg^{2+} ATPase purified from heavy sarcolemmal membrane previously treated with trypsin had several subunits with molecular weights of 90,000, 80,000, 67,000 and 20,000 (Tuana and Dhalla, 1987). Because the solubilization of the enzyme involved trypsin treatment, it is not clear if the lower molecular weight subunits originated from the proteolysis of the enzyme molecule or the 90,000 dalton peptide in the Ca^{2+}/Mg^{2+} ATPase (Tuana and Dhalla, 1987). It is interesting to note that each of the two subunits of the Ca^{2+}/Mg^{2+} ATPase reported in this study exhibited a molecular weight of about 90,000, which correspond to the largest subunit in the previoiusly purified Ca^{2+}/Mg^{2+} ATPase. In contrast to the Ca^{2+}/Mg^{2+} ATPase isolated from the light sarcolemmal fraction, the Ca^{2+}/Mg^{2+} ATPase purified from the heavy sarcolemmal membrane (Tuana and Dhalla, 1988) had higher activity with Ca^{2+} than with Mg^{2+} and its maximal specific activity (4-5 umol Pi/mg/min) was 15 - 25 fold lower. Despite the differences between two preparations of the Ca^{2+}/Mg^{2+} ATPase, it should be noted that these enzyme share several common characteristics. Both enzymes are activated by millimolar concentrations of Ca^{2+}, Mg^{2+} and other cations, and various inhibitors are found to exert similar effects on these two enzymes. Different purification procedures may not only yield enzyme proteins with different subunit structure and specific activities but may also yield enzyme preparations with different lipid and polysacharride compositions which may affect the activities of the membrane bound enzymes.

The plasma membrane Ca^{2+}/Mg^{2+} ATPases have also been isolated from rabbit skeletal muscle (Hidalgo et al., 1983; Kirley, 1988), human oat cell (Knowles and Leng, 1984) and sheep kidney medulla (Gantzer and Grisham 1979a,b). All these ATPases were activated by Ca^{2+} and Mg^{2+} with low affinity and hydrolyze other nucleoside triphosphates. Since these characteristics are similar to those for the rat heart Ca^{2+}/Mg^{2+} ATPase reported here, it is likely that all these ATPases may belong to a same family of the cell membrane Ca^{2+}/Mg^{2+} ATPase (Dhalla and Zhao, 1989). However, these ATPases seem to differ with respect to their molecular weights and subunit structures. The Ca^{2+}/Mg^{2+} ATPase from rabbit skeletal muscle transvers tubule was reported to shown two major subunits with molecular weights of 107,000 and 30,000(Hidalgo et al., 1983). Another report has indicated that Mg^{2+} ATPase from rabbit skeletal muscle may have a molecular weight of 105,000(Kirley, 1988). The Ca^{2+}/Mg^{2+} ATPase from sheep kidney medulla plasma membrane exhibited two peptides of 150,000 and 77,000 on SDS gel electrophoresis (Gantzer and Grisham, 1979a), while the enzyme from human oat cell plasma membrane contained a major protein band of 30,000 (Knowles and Leng, 1984). The Ca^{2+}-dependent ATPase from the rat heart heavy sarcolemmal membrane was reported to contain two subunits with molecular weights of 55,000 and 12,000 (Tuana and Dhalla, 1982). At present it is difficult to state with certainty whether the differences in the molecular structure of Ca^{2+}/Mg^{2+} ATPase from different sources are due to factors such as purification procedure or the methods used for the estimation of molecular weights or that these are isoenzymes with essentially similar properties.

The purified Ca^{2+}/Mg^{2+} ATPase from heart plasma membrane was found to be associated with a large amount (about 3,000 mole lipids per mole protein) of lipids. Although all of these lipids may not be important for the enzyme activity, it appears that the lipid environmet is vital for the enzyme activity. Phospholipase A, which hydrolyzes phospholipids to form lysophospholipids, and phospholipase C, which liberates diacylglycerol plus a phosphoryl base, decreased the Ca^{2+}/Mg^{2+} ATPase activity by changing the lipid environment of the enzyme. Furthermore, during purification study of the enzyme, it was observed that the enzyme was able to bind to ion-exchange column, concanavalin A affinity column, and hydrophobic column. However, little active Ca^{2+}/Mg^{2+} ATPase could be eluted from these columns (data not shown). This may be due to the loss of lipids upon the binding of the enzyme to the columns. The purification of the Ca^{2+}/Mg^{2+} ATPase in the presence of digitonin also resulted in an enzyme with little lipids and low ATPase activity. Although all major phospholipids existing in the heart plasma membrane were co-purified with the Ca^{2+}/Mg^{2+} ATPase, it should be noted that phosphatidylserine was especially enriched in the purified enzyme preparation. This may indicate a possible role of phosphatidylserine in maintenance or regulation of the Ca^{2+}/Mg^{2+} activity. Phosphatidylserine as a negatively charged lipid is also known to bind Ca^{2+} or other divalent cations and the Ca^{2+} bound to membrane phosphatidylserine and other negatively charged phospholipids has been suggested to be involved in the Ca^{2+} entry process during depolarization of the heart cell (Langer, 1978; Philipson et al, 1980). It is possible that the heart plasma membrane Ca^{2+}/Mg^{2+} ATPase may require phosphatidylserine bound Ca^{2+} or Mg^{2+} for its activity. Phosphatidylserine has also been reported to stimulate heart sarcolemmal Ca^{2+}-stimulated ATPase (Ca^{2+} pump ATPase) activity (Caroni et al., 1983). It should be pointed out that cholesterol was enriched in the purified Ca^{2+}/Mg^{2+} ATPase preparation to a lesser extent in comparison to phospholipids. Since animals fed high cholesterol diet, which is known to increase membrane cholesterol content, were found to exhibit higher Ca^{2+}/Mg^{2+} ATPase activity in heart sarcolemma (Moffat and Dhalla, 1985), we cannot exclude the possiblity that some tightly bound cholesterol may be important for the enzyme activity. Such a mechanism for the Ca^{2+}/Mg^{2+} ATPase would be different from that for the sarcoplasmic reticular Ca^{2+}-stimulated because the role of cholesterol was excluded from the phospholipid annulus surrounding Ca^{2+}-stimulated ATPase of sarcoplasmic reticulum (Warren et al., 1975). Nonetheless, the association of phospholipids with Ca^{2+}/Mg^{2+} ATPase purified from the heart plasma membrane appears to eliminate the need for reconstituting the purified enzyme into liposomes for studies on the physiological function of the Ca^{2+}/Mg^{2+} ATPase.

Because the Ca^{2+}/Mg^{2+} ATPase was found to be stimulated by a lectin concanavalin A, it was suggested that the enzyme was a glycoprotein (Zhao et al., 1989). In this study Con A decreased the ATP-induced rate of inactivation of the purified Ca^{2+}/Mg^{2+} ATPase. Furthermore, purified enzyme was observed to contain polysaccharides, which can be degraded by neuraminidase by 50%. Since the Ca^{2+}/Mg^{2+} ATP activity was inhibited by neuraminidase to a similar extent, it implies that polysaccharides may play an important role in the maintenance and/or regulation of the enzyme activity. Furthermore, the purified enzyme was stained positively by periodic acid - Schiff staining on electrophoresis gel and bound concanavalin A with a K_D similar to that of plasma membrane. Therefore, it is clear from this study that the Ca^{2+}/Mg^{2+} ATPase of heart plasma membrane is a glycoprotein. The skeletal muscle transverse tubule Mg^{2+} ATPase has also been suggested to be a glycoprotein because of its interaction with concanavalin A (Moulton et al., 1986).

From the foregoing discussion, it appears that the purified Ca^{2+}/Mg^{2+} ATPase preparation from heart plasma membrane is composed of a glycoprotein core with two peptides of 90,000 Da each and associated with plenty of lipids which are vital to the enzyme activity. The Ca^{2+} binding study has indicated that the purified Ca^{2+}/Mg^{2+} ATPase preparation can bind a large amount of Ca^{2+}. Since different other divalent cations displaced the Ca^{2+} bound to the Ca^{2+}/Mg^{2+} ATPase in an order similar to that for their abilities to inhibit the Ca^{2+} ATPase activity, it is evident that the Ca^{2+} binding may be required for initiating the activation of Ca^{2+}-ATPase activity. Although no efforts were made to quantify the proportion of Ca^{2+} bound to protein itself, the phospholipases A and C as well as neuraminidase decreased the Ca^{2+} binding. This indicated that at least part of the Ca^{2+} was bound to phospholipids and polysaccharide residues. It should be noted that Ca^{2+} bound to purified enzyme with a K_D (0.384 mM) similar to that of low affinity Ca^{2+} binding sites of heart sarcolemma (Gupta et al., 1989). The observed Ca^{2+} binding property of the purified Ca^{2+}/Mg^{2+} ATPase may support the view that the heart sarcolemmal Ca^{2+}/Mg^{2+} ATPase may be involved in the stimulus-induced Ca^{2+}-influx into the heart cell (Dhalla and Zhao, 1989; Dhalla et al., 1977a; Dhalla et al., 1984; Dhalla et al., 1985a). The opening of the slow channels through which the inward Ca^{2+} current traverses the heart sarcolemma has been shown to require metabolic energy (Sperelalakis and Schneider, 1976), and several energy yielding agents including ATP have been shown to produce an increase in contractile force of the cardiac muscle (Saks et al., 1977).

It has been suggested that Ca^{2+}/Mg^{2+} ATPase may be intimatedly involved in opening Ca^{2+} channels by phosphorylating these channels and this proposal is based on several biochemical and pharmacological observations. Since the extracellular concentration of Ca^{2+} is 1.25 mM, an enzyme system implemented in Ca^{2+} influx would have to be stimulated at millimolar concentrations. Various concentrations of Ca^{2+} have been reported to produce a linear corelation between the Ca^{2+}/Mg^{2+} ATPase activity and the heart muscle contractile force development (Dhalla et al., 1982). Gramicidin S which was found to inhibit the Ca^{2+}/Mg^{2+} ATPase also depressed the heart muscle contraction (Zhao and Dhalla, 1989). Drugs such as propranolol, quinidine, lidocaine and procainamide, and cations, such as Ni^{2+}, Co^{2+} and Mn^{2+}, which depressed Ca^{2+} influx, also decreased the activity of Ca^{2+} ATPase (Harrow et al., 1978; Dhalla et al., 1977b). In addition, phosphorylation of heart sarcolemma, which is associated with increased slow channel activity, stimulated the Ca^{2+}-ATPase activity (Ziegelhoffer et al., 1979). Furthermore, electrical stimulation of the rat heart sarcolemmal membranes in vitro was found to increase Ca^{2+}-ATPase activity; this effect was depressed by Ca^{2+}-antagonists verapamil and D600 (Ziegelhoffer and Dhalla, 1987). Although these observations are encouraging in defining the physiological role of Ca^{2+}/Mg^{2+} ATPase in heart function, a great deal of further research is needed to make any definitive conclusions.

It should be pointed out that the importance of extracellular ATP in regulating various biological processes has been recognized recently (Gordon, 1986). ATP has been shown to be released from heart cells (Clemens and Forrester, 1980), and various mammalian cells and several enzymes have been found to be capable of synthesizing ATP extracelluarly (Fenselan and Long, 1974; Packham et al., 1974; Sacks et al., 1977). The extracellular ATP and/or ATP bound to cell membrane can be seen to serve as a substrate for the sarcolemmal ATPase which, together with ADPase and 5'-nucleotidase, degrades ATP to adenosine, the latter is known to exert a wide variety of biological effects. The Ca^{2+}/Mg^{2+} ATPase may also participate in the termination of the ATP which has been proposed to serve as a purinergic neurotransmitter (Burnstock, 1981). Accordingly, the Ca^{2+}/Mg^{2+} ATPase can be considered to function as a purinergic receptor in the cell membrane. In this regard it may be noted that ATPrS bound to the purified Ca^{2+}/Mg^{2+} ATPase with two affinity sites with a Kd of about 50 nM and 1.2 uM. This binding was not due to the metabolism of the ATPrS because ATPrS is not a substrate for ATPases (Eckstein,1985). ATP has also been reported to bind to Na^{+}-K^{+} ATPase with a K_D of 0.2 uM (Hegyvary and Post, 1971) and to sarcoplasmic reticulum which was enriched with Ca^{2+}-stimulated ATPase with a K_D of 30 uM (Inesi et al., 1973). Since ATP was found to be able to regulate Ca^{2+}/Mg^{2+} ATPase activity in various cell membranes (Dhalla and Zhao, 1989), it is possible that one of the two binding sites, probably the high affinity binding site, may be a regulatory site while the other site is at the catalytic site. If this is true, the high affinity site of the enzyme may be regarded as an ATP receptor. However, much more work is needed to substantiate this view as well as to identify the role of ATP in the regulation of Ca^{2+}/Mg^{2+} ATPase and cardiac cell function.

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