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BOVINE BRAIN $\text{Ca}^{++}\text{Mg}^{++}$ ATPase:
PARTIAL CHARACTERIZATION

by James J. Valdes, Ph.D
Roy G. Thompson
Mia Paterno
Darrel E. Menking

RESEARCH DIRECTORATE

James P. Chambers, Ph.D.
Matthew J. Wayner, Ph.D.

UNIVERSITY OF TEXAS AT SAN ANTONIO
SAN ANTONIO, TX 78285

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Synaptic plasma membranes isolated from bovine brain exhibited a low and high affinity (Ca^{++} + Mg^{++})-dependent ATPase as evidenced by kinetic constants for ATP. One activity that hydrolyzed ATP maximally at pH 7.4 and 7.8 exhibited an eight-fold higher affinity when compared to the second or lower affinity activity that hydrolyzed ATP maximally at pH 7.0. Both activities exhibited submicromolar kinetic constants for Ca^{++} ($K_m = 0.24 \mu M$). K_m values for magnesium differed significantly. The lower activity affinity was approximately 6.5 times higher ($120 \mu M$) than that observed for the high affinity activity ($18 \mu M$). V_{max} values obtained under optimal assay conditions (low and high) were 110-135 and 43-55 nmol/min/mg protein, respectively. Both activities were KCN, NaN_3 , and ruthenium red insensitive. Only slight inhibition was observed in the presence of rotenone and oligomycin. Although both activities were observed to be trifluoperazine sensitive, they differed significantly with regard to other parameters. Na^+ and NH_4^+ ions preferentially inhibited the low affinity activity. (continued on reverse)					
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(Calcium (Ca^{++}) + Magnesium (Mg^{++}))

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19. Abstract (continued)

greater than 90%. Cs^+ ions completely inhibited the high affinity activity but reduced the low affinity only 22%. Li^+ , Al^{+3} and Mn^{++} significantly inhibited the high affinity activity but reduced the low activity only moderately. The low and high affinity were inhibited by vanadate with half-maximum inhibition occurring at 2 and 5 μM , respectively, indicating the plasma membrane origin of these activities. Thermal denaturation studies indicated that the high affinity activity was stable for 2 min at 45 $^{\circ}\text{C}$ after 50% of the activity was lost at 2.5 min. In contrast, the low affinity activity gradually decreased over the time course and retains greater than 60% activity at 2.5 min. The positive allosteric calcium channel modulator, diltiazem, stimulates both low and high affinity activities, 10 and 40% at 10 and 30 μM , respectively. In contrast, the negative allosteric calcium channel modulator, verapamil, has no effect upon the high affinity activity while slightly inhibiting the low affinity activity. These observations are suggestive of either a direct interaction with the $(\text{Ca}^{++} + \text{Mg}^{++})$ -dependent ATPase or a close spatial relationship of either a specific catalytic site of conformation of the ATPase with that of the diltiazem binding site or resultant conformation of the calcium channel following diltiazem administration. Although kinetic data are consistent with other findings that indicate the presence of different kinetic conformations of a single synaptic membrane protein, we can not rule out the presence of two different proteins maximally operational at two different ATP concentrations. *Keywords: adenosine phosphates; (AT).*



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PREFACE

The work described in this report was authorized under Project No. 1L162706A553, CB Defense and General Investigation, Decontamination, Detection, and Identification. This work was started in June 1984 and completed in September 1986. The experimental data are contained in laboratory notebooks in the Division of Life Sciences, University of Texas at San Antonio.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the committee on Revision of the Guide for Laboratory Animals Resources, National Research Council.

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BOVINE BRAIN $\text{Ca}^{++}\text{Mg}^{++}$ ATPase: PARTIAL CHARACTERIZATION

1. INTRODUCTION

The function of Ca^{+2} extruding ATPases is to maintain the internal free Ca^{+2} concentrations around or below the micromolar level. These ATPases exhibit high affinity for Ca^{+2} as expressed by K_m values in the range 0.1-0.5 μM , and are apparently intrinsic membrane proteins because various detergents extracted them in soluble form. Purifications of many of these solubilized preparations were completed by transport-specific fractionation^{1,2} and calmodulin affinity chromatography.³⁻⁷

It is not yet clear whether these membrane transport proteins function in the membrane as monomers or in an aggregated multimeric state. Structural studies suggest that the ATPase peptides of the sarcoplasmic reticulum are in close contact in the membrane.^{8,9} Radiation inactivation studies suggest proximity between Ca^{+2} -ATPase molecules in a dimeric complex.¹⁰ Studies of bidimensional membrane crystals, formed in the presence of vanadate, show that the minimal asymmetric unit using these conditions consists of a dimer.¹¹ Martins and de Meis observed in studies of partial reaction of soluble and membrane-bound sarcoplasmic reticulum ATPase impairment of different intermediate reactions of the catalytic cycle.¹² Other studies show that a detergent solubilized monomer of ATPase preparations exhibits an activity similar to that of the native enzyme.¹³⁻¹⁵

Although various ATPase reconstitution studies using protein from different sources have partially elucidated phospholipid requirements, there is evidence that suggests membrane components such as cholesterol influence the function of numerous membrane proteins (e.g. Na^{+} , K^{+} -ATPase,¹⁶ acetylcholine receptor,¹⁷ and band 4.5 glucose transport protein¹⁸). Recently, inhibitor and activator proteins of a Ca^{+2} ATPase in rat liver plasma membranes were observed,¹⁹ and a 53,000 dalton glycoprotein that enhances the ATPases' affinity for calcium^{20,4} was isolated from the sarcoplasmic reticulum.

Few reports characterize Ca^{+2} + Mg^{+2} -dependent ATPase in situ, using native synaptic membrane preparations. To obtain basic information about the properties of the Ca^{+2} + Mg^{+2} -dependent ATPase that would guide and support future studies, we studied the properties of Ca^{+2} + Mg^{+2} -dependent ATPases of bovine brain synaptic membranes. This tissue is a good source of protein due to its size and easy availability. In this report, we present evidence that suggests the presence of two kinetic forms of Ca^{+2} + Mg^{+2} -dependent ATP hydrolase activity from bovine brain.

2. MATERIALS AND METHODS

2.1 Materials.

Adenosine-5'-triphosphate, from rabbit muscle, and trifluoperazine were obtained from Boehringer-Mannheim. HEPES, tris, ouabain, oligomycin, diltiazem and verapamil were obtained from Sigma Chemical Company, St. Louis, MO. Malachite green was purchased from Aldrich Chemical Company, Milwaukee, WI. All general laboratory reagents were of highest grade.

2.2 Preparation.

Synaptosomes were prepared from approximately 10 g of bovine brain tissue (cortex) obtained from Roeglein Provision Company, San Antonio, TX, approximately 15 min after exsanguination. Synaptosomes were prepared by the method of Hajos.²¹ Bradford's²² method of using bovine serum albumin as standard determined the protein.

2.3 ATPase Assay.

Phosphate released by hydrolysis of ATP was monitored spectrophotometrically by the method of Lanzetta and co-workers.²³ All incubations contained 100 μ g of synaptic membrane protein in a final volume of 2.0 mL, 0.01 M HEPES buffer at designated pH, calculated amounts of EGTA and/or EDTA, 0.3 mM ouabain, and indicated amounts of ATP. Reactions were carried out for 90 sec and terminated by addition of 200 μ L 6N HCl. After thorough mixing, a 200 μ L aliquot was removed, and the released phosphate was determined spectrophotometrically. Total ATPase activity was determined in the presence of indicated amounts of free Mg^{+2} and Ca^{+2} ions. $Ca^{+2} + Mg^{+2}$ dependent ATPase activity was determined by taking the difference between assays run in the presence of both Mg^{+2} and Ca^{+2} ions and those run only in the presence of Mg^{+2} . All assays were conducted in triplicate with appropriate blanks. The amount of EGTA added to the buffer to control free Ca^{+2} in the presence of Mg^{+2} was calculated according to Bartfai.²⁴ To check the dependency of hydrolysis of ATP on free Mg^{+2} , we calculated the amount of $MgCl_2$ to be added in the presence of buffer that contained EDTA according to the formulas and constants employed by Pershadsingh and McDonald.²⁵ Two different assays referred to as "low" and "high" affinity were used with respect to ATP concentrations where indicated. Low affinity assays were conducted in 0.01 M HEPES buffer, pH 7.0, and high affinity assays were conducted in 0.01 M HEPES buffer, pH 7.4. All assays contained 0.3 mM ouabain. Table 1 summarizes the low and high affinity assay conditions.

Table 1. Reaction Micromolarity

<u>Low Affinity</u>		<u>High Affinity</u>
ATP	200	12.5
Mg^{+2}	250	100
Ca^{+2}	2.52	2.52

2.4 Analysis of Data.

All kinetic data were analyzed by the Eadie-Hofstee graphic procedure as described by Walter.²⁶ This type of graphic analysis was the most sensitive to deviation from hyperbolic enzyme kinetics.²⁶

3. RESULTS

Two regions of saturation were observed with regard to release of phosphate from ATP when assayed at a final hydrogen ion concentration of 7.2 in the presence of 2.52 μM free Ca^{+2} and 200 μM free Mg^{+2} (Figure 1). One, a high affinity activity, saturated at very low ATP concentrations (approximately 15 μM). A second, but lower affinity ($\text{Ca}^{+2} + \text{Mg}^{+2}$ -dependent ATPase activity, saturated at higher ATP concentrations (approximately 40-45 μM). In an attempt to further characterize these activities, we examined the dependence of these activities on hydrogen ion concentration. Two distinct profiles were observed depending upon the ATP concentration used in the assay (Figure 2). When assayed in the presence of 200 μM ATP (x-x), hydrolysis of ATP occurred over a broad range of hydrogen ion concentration with maximum hydrolysis at pH 7.0. Assays conducted in the presence of high ATP concentration (12.5 μM , 0-0) also indicated a broad pH dependence with one maxima at pH 7.4 and a second at pH 7.8. Assay of synaptic membrane homogenate preparations at pH 7.0 in the presence of higher and increasing concentrations of ATP (25-125 μM), and graphical analysis of the ratio of velocity, and substrate concentration versus that of velocity indicated K_m and V_{max} values of 24 μM and 110 $\mu\text{moles/min/mg}$ protein, respectively (Figure 3). Synaptic membrane homogenates assayed at pH 7.4 in the presence of low but increasing concentrations of ATP (2.5 - 15 μM) indicated saturation to occur at approximately 7.5 μM (Figure 4). Eadie-Hofstee graphical analysis of these data shown in the inset indicated K_m and V_{max} values of 3.2 μM and 56 $\mu\text{moles/min/mg}$ protein, respectively. Evaluation of the dependence of hydrolysis of ATP on free calcium at low (12.5 μM) and high (200 μM) ATP concentrations buffered at optimal hydrogen ion concentration is shown in Figure 5, Frame A, with an Eadie-Hofstee graphical analysis in Frame B. Hill Plot analysis (Figure 5, Frame C) indicated slopes of 1.1 and 0.5 under low and high affinity assay conditions, respectively. Both catalytic activities exhibited a K_m value of 0.24 μM for calcium. However, V_{max} values obtained at low and high ATP concentrations differed significantly (128 and 49 $\mu\text{moles/min/mg}$ protein). Figure 6 shows the dependence of hydrolysis of ATP upon magnesium. Eadie-Hofstee graphical analysis (high and low ATP concentrations, inset) revealed K_m values of 18 and 112 μM , respectively. Maximum velocity values were similar to those obtained for previously examined kinetic parameters. Table 2 summarizes the kinetic constants assayed under optimal hydrogen ion, free calcium and magnesium, and ATP concentrations. These conditions are referred to as low and high affinity assay conditions. To ascertain if these activities were mitochondrial in nature, low and high affinity ATPase assays were performed in the presence of several mitochondrial inhibitors (Table 3). As indicated in the table, KCN, NaN_3 , and ruthenium red did not inhibit either activity. Rotenone and oligomycin, in general, inhibited 10-15%.

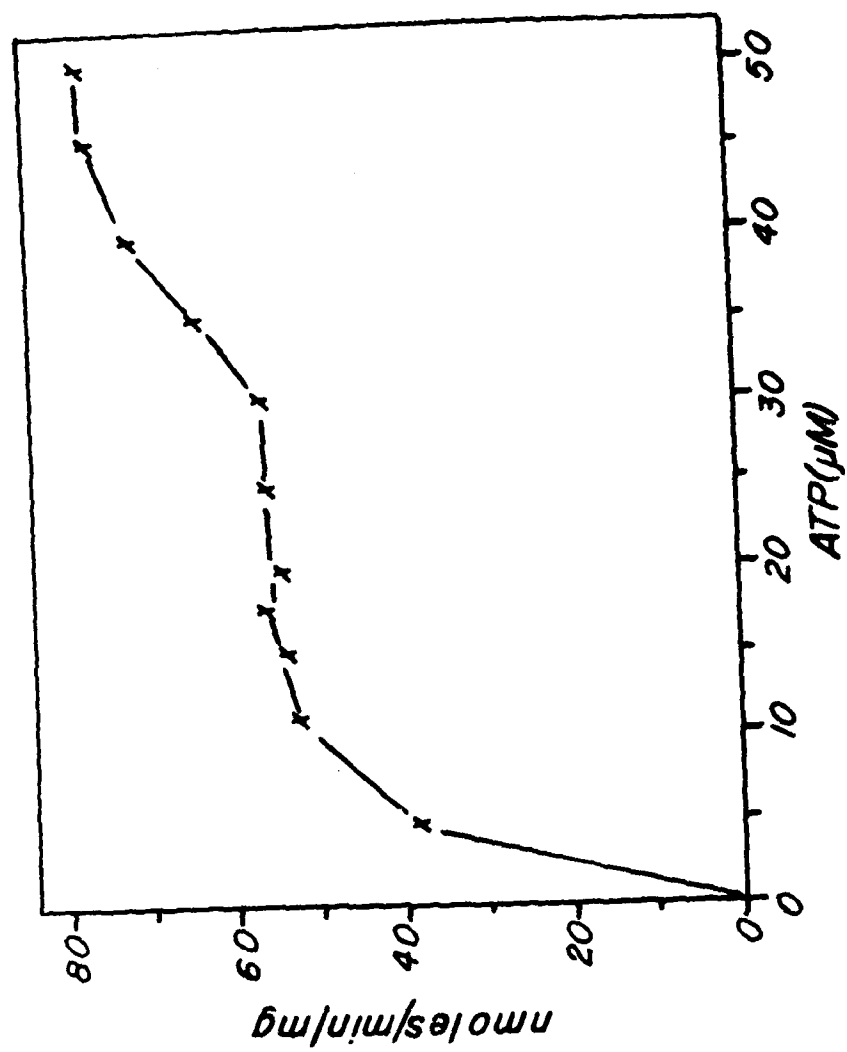


Figure 1. Ca^{+2} + Mg^{+2} -Dependent ATP Hydrolysis as a Function of ATP Concentration

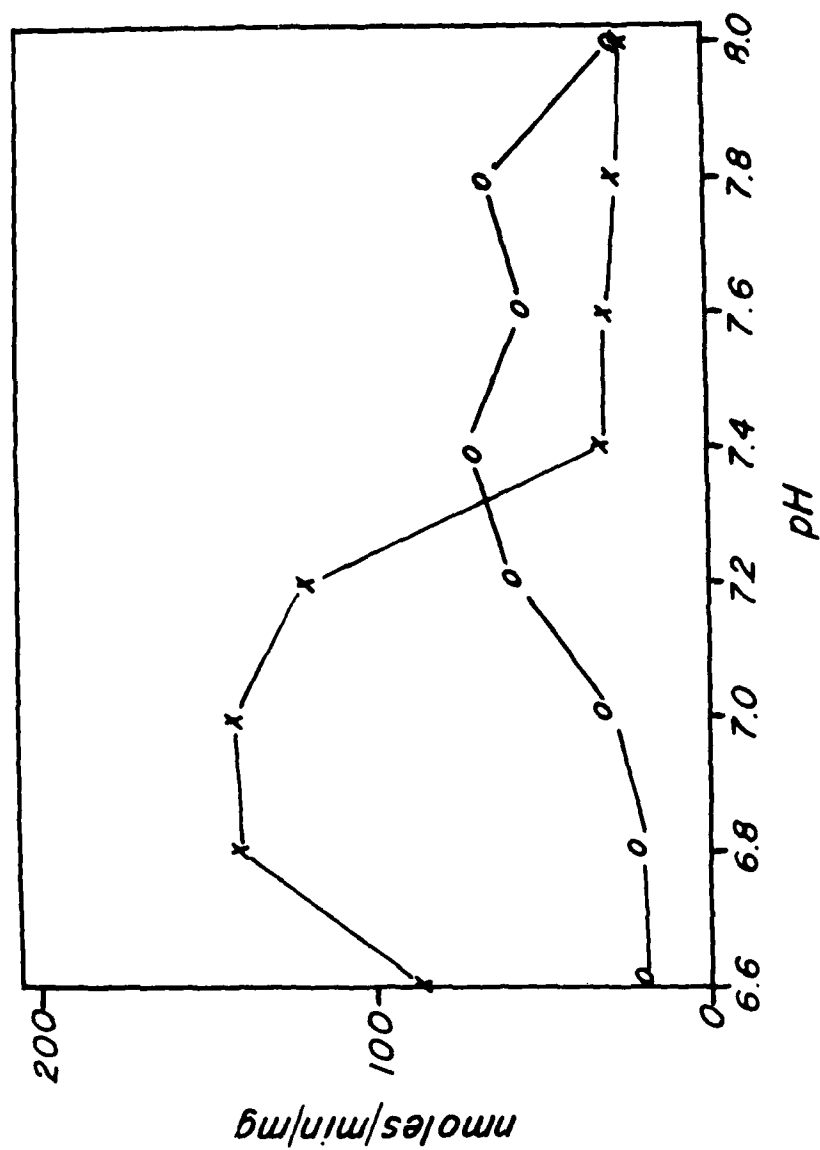


Figure 2. Dependence of Hydrolysis of ATP on Hydrogen Ion Concentrations

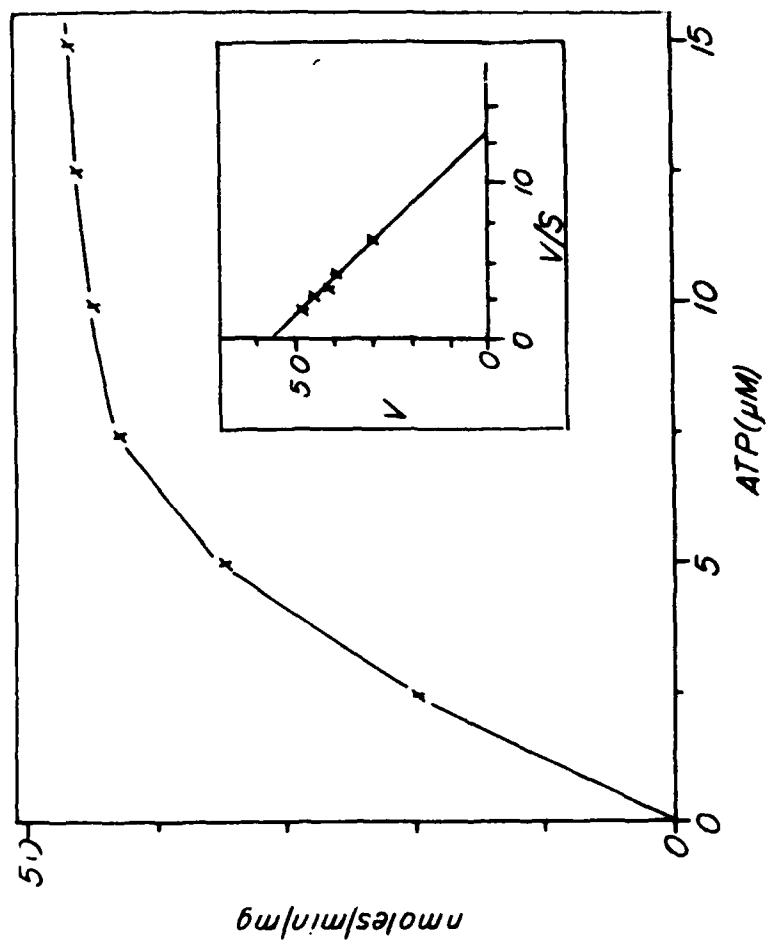


Figure 3. $\text{Ca}^{+2} + \text{Mg}^{+2}$ -Dependent ATPase (High Affinity) Activity as a Function of Increasing ATP Concentration

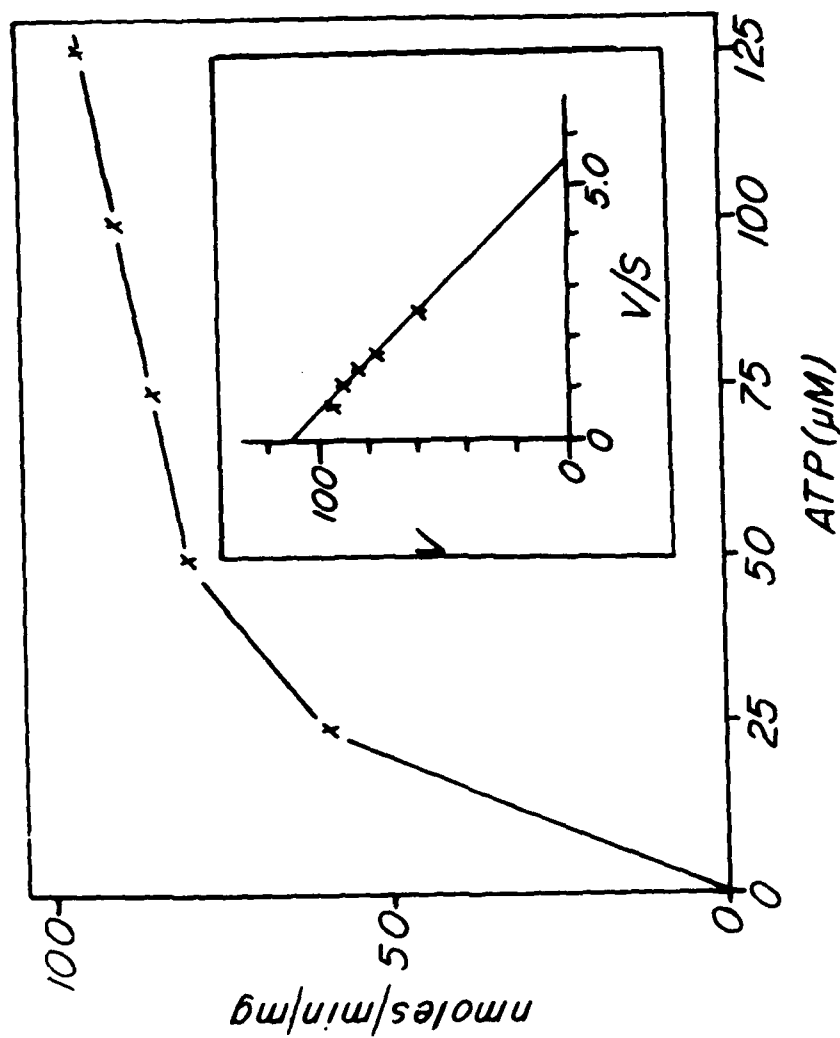


Figure 4. Ca^{+2} + Mg^{+2} -Dependent ATPase (Low Affinity) as a Function of Increasing ATP Concentration

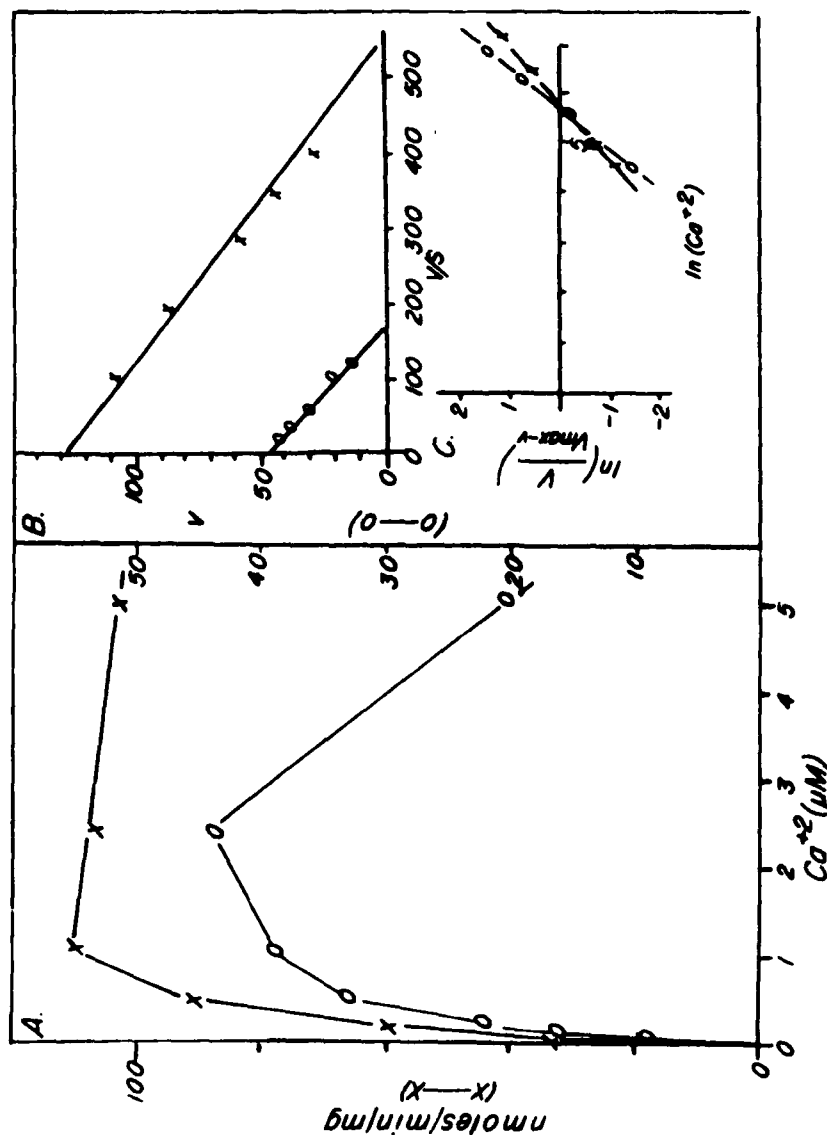


Figure 5. $\text{Ca}^{+2} + \text{Mg}^{+2}$ -Dependent ATPase Activity as a Function of Free Ca^{+2} . A. All Incubations were carried out under either Low (x-x) or High Affinity (o-o) Assay Conditions with Free Ca^{+2} varied as Indicated in the Figure. B. Eadie-Hofstee Graphical Analysis of Kinetic Data. C. Hill plot Analysis of Kinetic Data.

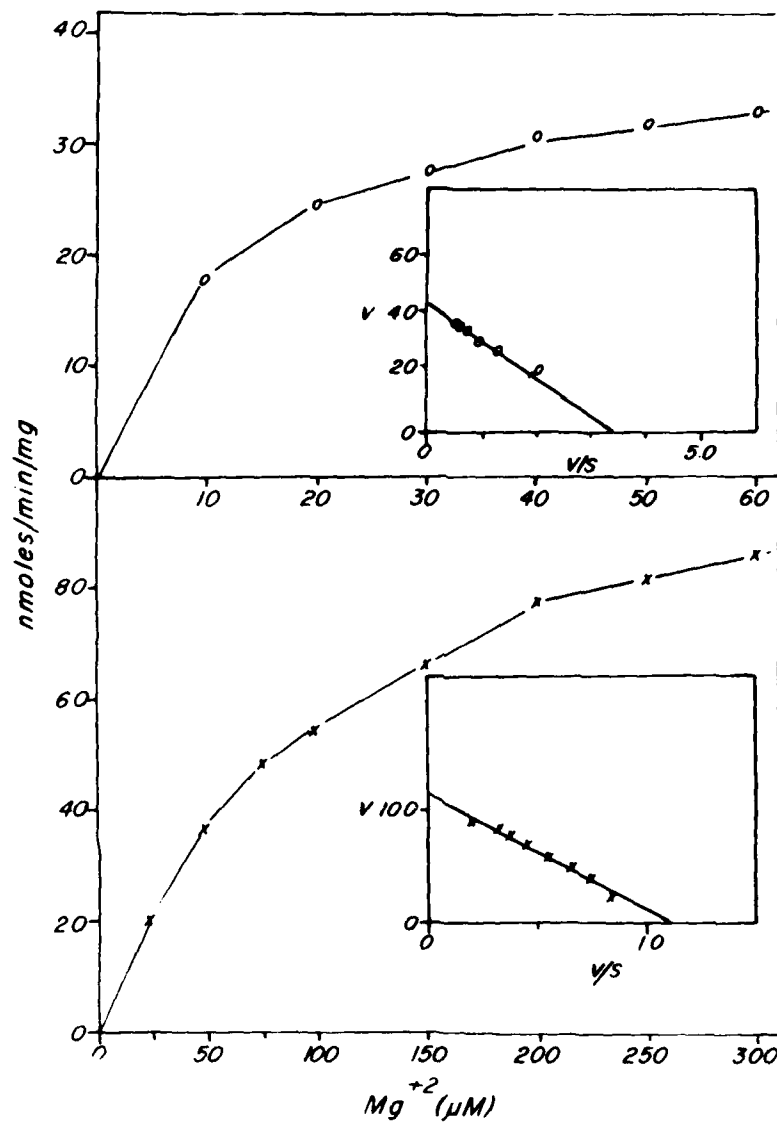


Figure 6. $Ca^{+2} + Mg^{+2}$ -Dependent ATPase Activity as a Function of Free Mg^{+2} .

Table 2. Summary of Kinetic (K_m) Constants

	(μM)	
	<u>Low</u>	<u>High</u>
ATP	24	3.2
Ca ⁺²	0.24	0.24
Mg ⁺²	110	18

Table 3. Effects of Various Cations on Low and High Affinity ATPase Activities from Bovine Brain Synaptosomes

	(% Inhibition)	
	<u>Low</u>	<u>High</u>
Rotenone (12μM)	15	20
KCN (0.1mM)	0	0
NN ₃ (0.1mM)	0	0
Oligomycin (50μM)*	15	10
Ruthenium red (10μM)	0	0

* Micromolarity calculated on an average molecular weight of 786.

To determine if one or both of these activities were calmodulin mediated, synaptic membrane homogenates were assayed under low and high affinity conditions in the presence of increasing amounts of the known calmodulin antagonist, trifluoperazine. Both activities were inhibited maximally (approximately 60%) at 30 μM trifluoperazine (Figure 7). The low affinity activity was very sensitive to the classical inhibitor vanadate (Figure 8), whereas the high affinity activity required approximately twice as much inhibitor to reduce the activity to 50% of control. In the presence of 10 μM vanadate, both activities were reduced 90% or more. Lanthanum reduced both activities similarly but was not as effective at lower concentrations. An extensive list of various cations (mono, di, and trivalent), and their effect upon ATP hydrolysis is shown in Table 4. Low and high affinity activities were inhibited by the cations to various degrees. However, sodium and ammonium ions preferentially inhibited greater than 90% of the low affinity activity but left greater than 90% of the high affinity activity intact. Cesium, aluminum, and manganese totally inhibited the high affinity enzyme. Manganese and aluminum ions inhibited low affinity activity approximately 50%, whereas cesium resulted in stimulation.

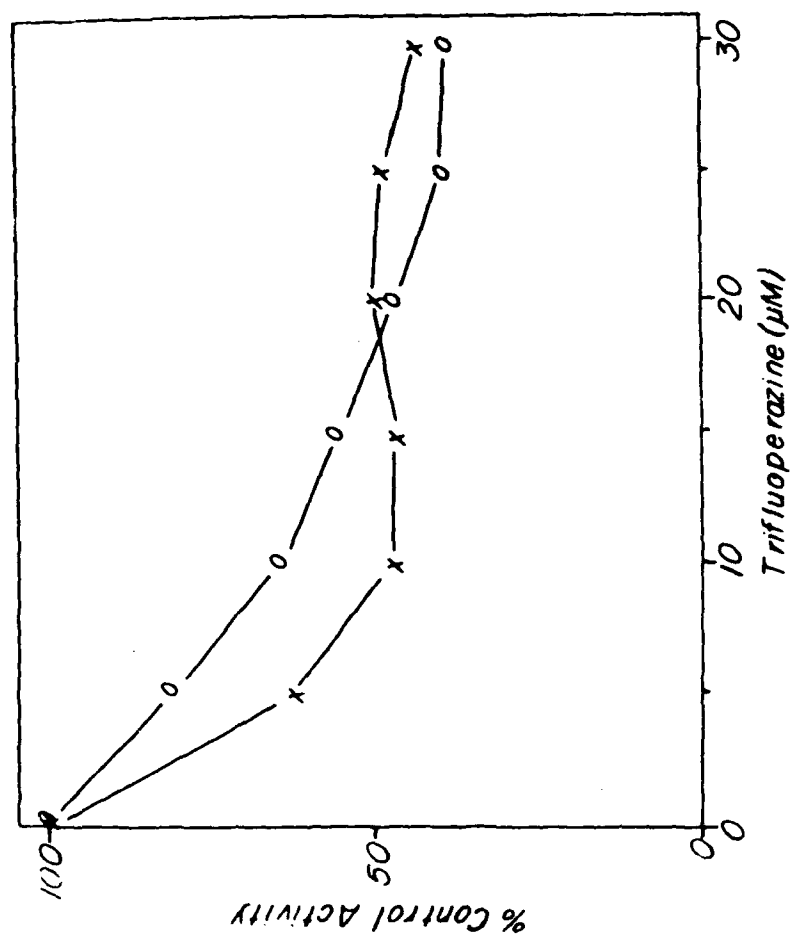


Figure 7. Effects of Trifluoperazine on Low (x-x) and High (o-o) Affinity ATPase Activities from Bovine Brain Synaptic Membrane Homogenates.

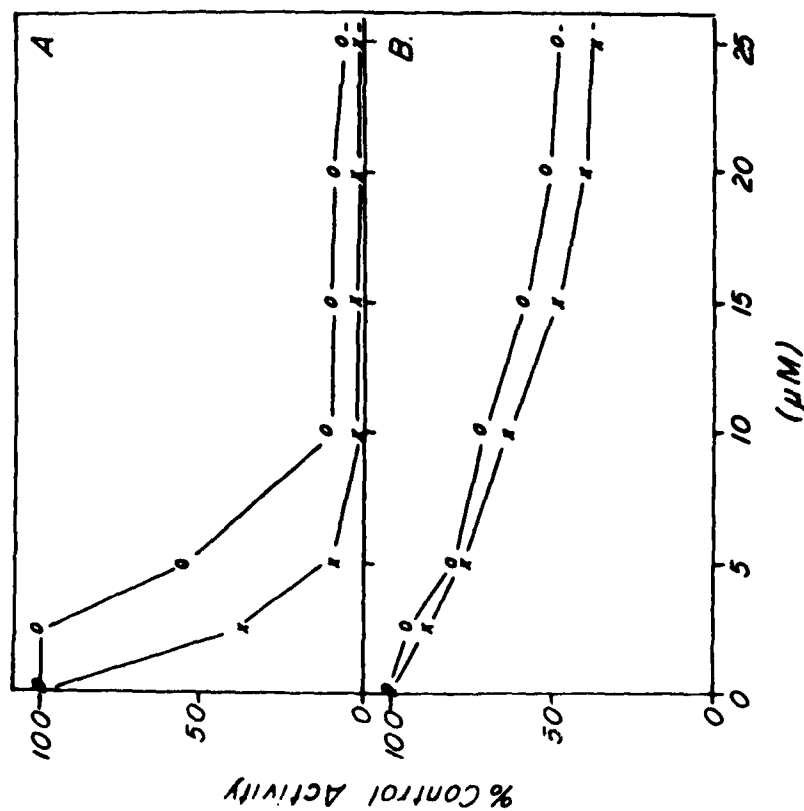


Figure 8. Effects of Vanadate and Lanthanum on Low and High Affinity ATPase Activities from Bovine Brain Synaptic Membrane Homogenates.

Table 4. Effects of Various Mitochondrial Inhibitors on Low and High Affinity ATPase Activities from Bovine Brain Synaptic Membrane Homogenates

	Low Affinity % Control	High Affinity % Control
Cs ⁺ 1	78	0
Na ⁺ 1	5	91
NH ₄ ⁺ 1	9	94
Li ⁺ 1	57	11
Mn ²⁺	52	0
Cu ²⁺	56	66
Zn ²⁺	71	58
Co ²⁺	34	34
Ba ²⁺	72	132
Fe ³⁺	49	28
Al ³⁺	51	0

A thermal lability study revealed the two activities to exhibit differences with regard to thermal denaturation (Figure 9). The low affinity activity gradually lost activity when incubated at 45 °C for increasing periods of time. In contrast, the high affinity activity remained constant and unaffected for 2 min after approximately 50% activity was lost. We were interested in determining the effects of the allosteric calcium channel modulators, verapamil and diltiazem, on these ATPase activities. Diltiazem significantly stimulated the high affinity ATPase activity but only slightly stimulated (approximately 10%) the lower affinity activity (Figure 10). The slight stimulation was followed by a gradual but significant decline in ATP hydrolysis. Verapamil inhibited only the low affinity activity with no apparent effect upon the high affinity ATPase activity (Figure 10).

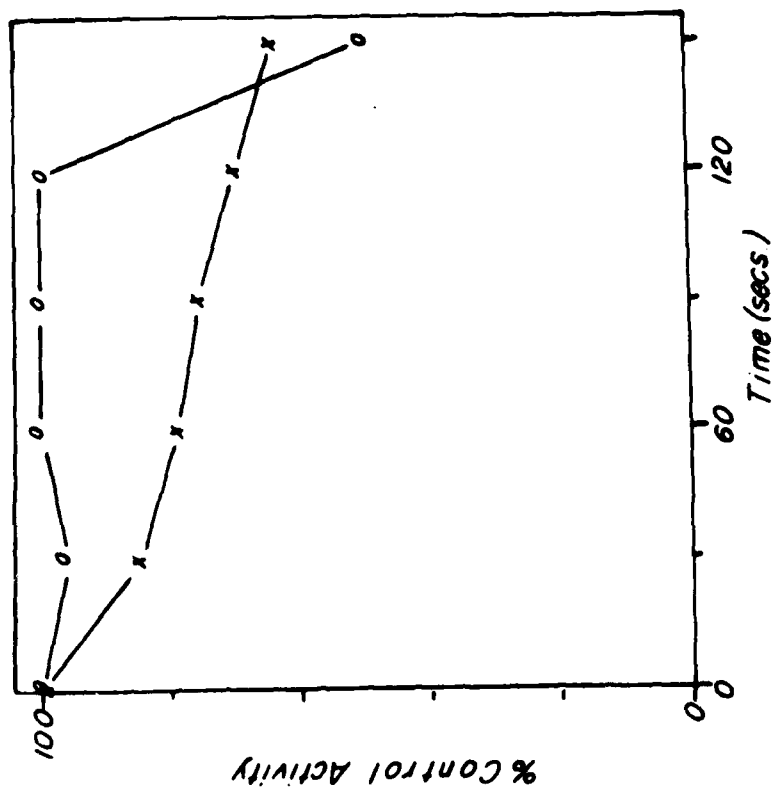


Figure 9. Thermal Lability of Low and High Affinity ATPase Activities from Bovine Brain Synaptic Membrane Homogenates.

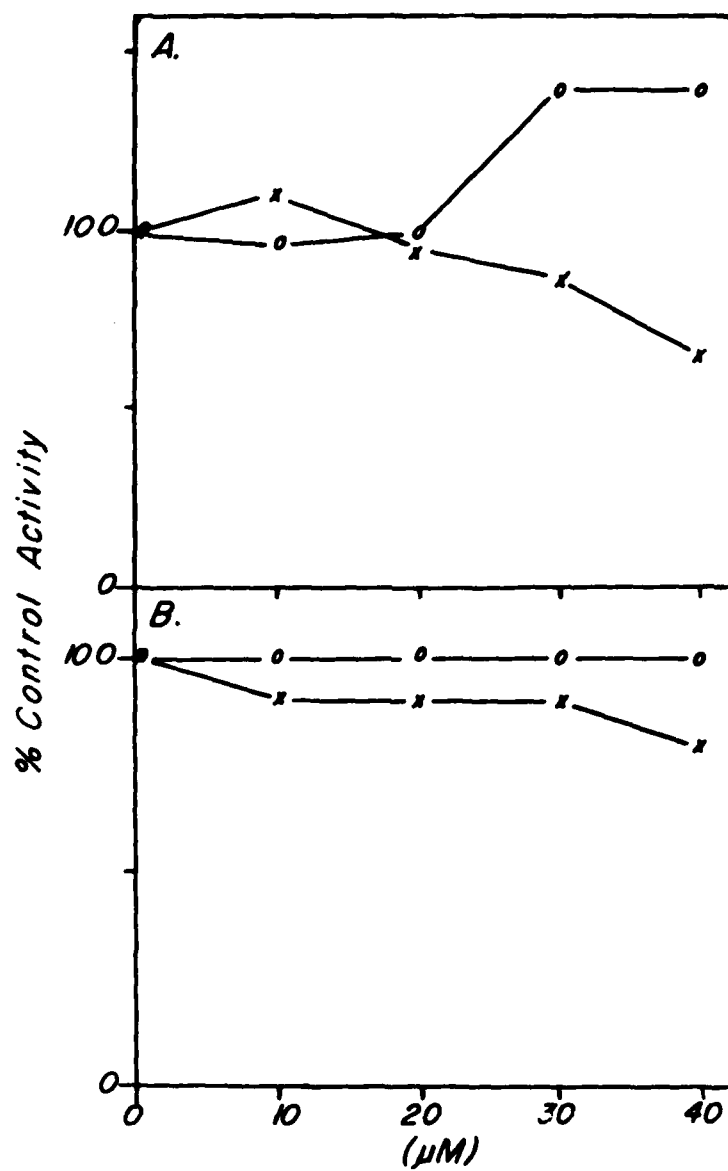


Figure 10. The Effects of Diltiazem and Verapamil on Low and High Affinity ATPase Activities from Bovine Brain Synaptic Membrane Homogenates.

4. DISCUSSION

The evidence suggests the presence of two kinetic forms of $\text{Ca}^{+2} + \text{Mg}^{+2}$ -dependent ATP hydrolase activities in bovine brain synaptic membrane homogenates that could be distinguished on the basis of different pH profiles and saturation plots using ATP as substrate. The pH studies were performed in the presence of a fixed amount of total calcium in the presence of EGTA. The calcium/EGTA association constant is strongly pH-dependent.²⁴ Measurement of free Ca^{+2} over the narrow pH range used in this study revealed no changes in free Ca^{+2} concentration (data not shown). Earlier studies, using sarcoplasmic reticulum vesicles, revealed the presence of a high affinity ATP binding site with a K_m of 1-5 μM that, when phosphorylated in the presence of Ca^{+2} , gave rise to a low-affinity ATP-binding site with a K_m of 30-500 μM accompanied by a twofold to threefold increase in V_{max} .²⁷

Recently, Villalobo, et al., studied the Ca^{+2} -translocating ATPase from human erythrocytes plasma membrane and observed a complex interaction between the major ligands (i.e., Ca^{+2} , Mg^{+2} , H^{+} , calmodulin and ATP) and the enzyme.²⁸ Furthermore, they demonstrated that the catalytic cycle of the Ca^{+2} -translocating ATPase maintains multiple binding sites for Ca^{+2} and two affinities for ATP, depending upon the presence or absence of calmodulin. Effects of calmodulin removal was not directly examined, but under the described reaction conditions, the calmodulin antagonist trifluoperazine inhibited approximately 60% of both ATPase activities (Figure 7). Some of the calmodulin is possibly inaccessible and is not affected by trifluoperazine treatment.

Although the Villalobo studies used enzyme preparations derived from nonneuronal tissues, there appear to be common features with synaptic membrane bound enzyme. For example, under optimal assay conditions, the high affinity activity exhibits a K_m of 3.2 μM with a maximum velocity of 43-55 nmoles Pi released/min/mg protein. The low affinity activity exhibited a K_m for ATP of 24 μM and a corresponding maximum velocity of 110-135 nmoles Pi released/min/mg protein, which essentially represents a twofold increase in V compared to the high affinity activity. Examination of Ca^{+2} dependence revealed 2.52 μM free Ca^{+2} to be optimal with a decrease in high affinity activity at higher free Ca^{+2} concentration. Although the K_m values derived under low and high ATP concentrations were identical (0.24 μM), Hill coefficients were not, indicating no interaction (cooperativity) of Ca^{+2} under low affinity conditions. Thus, a single binding site for Ca^{+2} or multiple binding sites that are completely independent of one another exist under low affinity conditions. Under high affinity conditions, we observed a Hill constant of <1 , which indicates a negative cooperativity between Ca^{+2} and more than one Ca^{+2} binding site. Using a calmodulin-affinity purified preparation, Villalobo and co-workers observed much higher Hill coefficients that indicated significant positive cooperativity between Ca^{+2} binding sites. These differences could arise from either the differences in tissue as a source of protein or the differences in molecular environment arising for detergent solubilization. Additionally, steady-state kinetics of ATP hydrolysis reflect different Hill coefficients, depending on the pH and concentrations of Na^{+} , K^{+} , and Mg^{+2} .²⁹⁻³³ Michaelis and co-workers, using synaptic membranes from mammalian brain tissue, observed $K_{0.5}$ (Ca^{+2}) of 0.23, $K_{0.5}$ (Mg^{+2}) of 6.6 μM , and K_m for ATP of 18.9 μM .³⁴ With the exception of the kinetic constant for Mg^{+2} , these values are in general agreement with those we derived for the low affinity activity.

The two affinities we describe possibly represent two different membranes (i.e., plasma versus microsomal). The low and high affinity activities we describe were very sensitive to vanadate and, to a lesser degree, lanthanum. Some investigators used vanadate sensitivity to discriminate microsomal and plasma membrane $\text{Ca}^{+2}+\text{Mg}^{+2}$ -dependent ATPase activity from that of the sarcoplasmic reticulum.³ Half-maximal inhibition of the low and high activity by vanadate is approximately 2 and 5 μM , respectively. Evaluation of effects of various mono, di, and trivalent cations indicated the monovalent cations, sodium and ammonium, preferentially inhibited the low affinity activity. Previously, Lotersztajn and Pecker observed various cations to uncouple Ca^{+2} transport from Ca^{+2} stimulated $(\text{Ca}^{+2}+\text{Mg}^{+2})$ -ATPase in rat liver plasma membrane.¹⁹ In that study, cations were carefully substituted for CaCl_2 . Our data do not represent a substitution but merely a determination of the effects of addition of various cations, and the free cationic concentration is probably considerably lower than the added 25 μM . However, the free cationic concentration is probably considerably higher than those "free" cation concentrations used in the Lotersztajn and Pecker study. The possibility that cations have not effectively increased the free Ca^{+2} concentration by combining with EGTA, can not be ruled out; although, no stimulation by Fe^{+2} , Mn^{+2} or Co^{+2} in the rat liver plasma membrane was observed.¹⁹ Na^{+1} -sensitive Ca^{+2} transport is a recognized component of plasma membranes from excitable tissues³⁵ and has also been described in kidney³⁶ and dog red blood cells.² We found Na^{+} as well as NH_4^{+1} ions to be significantly inhibitory. Lin and Way concluded that Mg^{+2} , Na^{+2} , K^{+} , and ATP have specific roles in regulating Ca^{+2} permeability of the plasma membrane, calcium binding, and calcium extrusion.³⁷ In addition to the preferential effects of Na^{+1} and NH_4^{+1} ions, Cs^{+1} , Mn^{+2} , and Al^{+3} completely inhibited the high affinity activity but reduce the low affinity activity 22, 48, and 49%, respectively. If the low and high affinity ATPase activities we describe represent two different configurations as previously proposed,²⁷ a certain configuration could interact with specific charge groups that may be inaccessible in the alternate configuration. Examination of thermal lability revealed that the high affinity activity appears to be heat stable up to 2 min then loses activity rapidly. The low affinity activity lost activity gradually over the duration of the experiment.

The 1,4-dihydropyridine family of calcium antagonists binds saturably and reversibly with high affinity to specific sites that appear to mediate blockade of calcium ion flux through voltage-dependent calcium channels in a number of tissues.³⁸ Binding studies with radioactive derivatives have shown that the dihydropyridine derivatives recognize a binding site distinct from that of verapamil and diltiazem.³⁹⁻⁴¹ Diltiazem (30 μM) stimulated high affinity ATP hydrolysis 40% and low affinity activity 10% at lower concentration. Verapamil, the negative allosteric calcium channel modulator, had no effect upon the high affinity ATPase activity, whereas a slight decrease was observed under low affinity assay conditions. These results raise the possibility that the calcium channel allosteric sites and the low affinity ATP catalytic conformations or site may be located very close to one another or that the conformations that result from diltiazem administration may be more optimal for ATP hydrolysis.

Recent reports by Lin and Way⁴² and Papazian et al.⁴³ suggest that the Ca^{+2} ATPase, Mg^{+2} -ATPase, and $(\text{Ca}^{+2}+\text{Mg}^{+2})$ -ATPase activities in synaptic plasma membranes reflect the operation of three separate enzymes. The $(\text{Ca}^{+2}+\text{Mg}^{+2})$ -ATPase activity is thought to represent enzyme activity linked to a Ca^{+2} transport process in nerve endings.^{37,44} Lin and Way conclude that synaptic plasma membrane contains a high affinity Ca^{+2} -ATPase that may have a functional role in the

removal of cytosolic Ca^{+2} .⁴⁵ The activities we describe represent only the $(\text{Ca}^{+2}+\text{Mg}^{+2})$ -ATPase.

The detailed enzyme mechanism that couples ATP hydrolysis and ion pumping is not well understood. In 1982, Hammes proposed, in a method similar to Fick,²⁷ a single mechanism that describes coupling between ion transport and catalysis for a diverse group of enzymes.⁴⁶ For the proposed mechanism to work, the enzyme must have two available conformations. Formation of the phosphoenzyme by ATP occurs with one conformation of the enzyme, and hydrolysis of the phosphoenzyme occurs with the other conformation of the enzyme. An interaction among major ligands (ie., Ca^{+2} , Mg^{+2} , H^{+1} , calmodulin and ATP) and enzyme may exist.²⁸ Although the structural basis of conformational changes associated with the ion pumping mechanism is the subject of numerous studies in the sarcoplasmic reticulum⁴⁷⁻⁵¹, much more information is needed on the synaptic processes.

CONCLUSIONS

Our results suggest that the Ca^{++} channel either directly interacts with the $(\text{Ca}^{++} \text{ Mg}^{++})$ -dependent ATPase, or that a close spatial relationship exists between a specific catalytic site of the ATPase in either the diltiazem binding site or the resultant conformational change in the Ca^{++} channel. Although these results are consistent with the hypothesis of two kinetic conformations of a single synaptic membrane protein, we cannot rule out the alternative hypothesis that there are two different concentrations.

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