High affinity Ca$^{2+}$-stimulated Mg$^{2+}$-dependent ATPase in Rat Brain Synaptosomes, Synaptic Membranes, and Microsomes*

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The regulation of intraneuronal free calcium ion concentrations, [Ca$^{2+}$]$_i$, within the range of 0.1–1.0 μM has been a topic of intensive investigation because of the importance of this ion in the control of neuronal excitability and transmitter release (e.g. 1–4). Some of the processes suggested to be involved in the regulation of [Ca$^{2+}$]$_i$ include the transport of Ca$^{2+}$ into mitochondria (5, 6), the binding of Ca$^{2+}$ by intraneuronal or membrane proteins (7, 8), the exchange of intraneuronal Ca$^{2+}$ with extracellular Na$^+$ through a Na$^+$/Ca$^{2+}$ antiport process (9–11), and finally, Ca$^{2+}$ extrusion or sequestration by Ca$^{2+}$-activated ATPases (5, 12–15).

The ATP-dependent transport of Ca$^{2+}$ has been viewed by some investigators as a sequestration of Ca$^{2+}$ within vesicular structures present in nerve endings (5, 12, 13, 22, 23). It has been proposed by others, however, that ATP-dependent Ca$^{2+}$ transport in nerve terminal preparations (synaptosomal membranes) is a plasma membrane process that leads to Ca$^{2+}$ extrusion from the intra- to the extraneuronal space (14, 18, 19, 21, 24). The Ca$^{2+}$-stimulated Mg$^{2+}$-ATPases in microsomal and synaptosomal membrane fractions have been studied previously to determine the characteristics of the enzymes which apparently mediate the ATP-dependent Ca$^{2+}$ transport. Both synaptosomal membrane preparations and microsomal membranes have been shown to have (Ca$^{2+}$ + Mg$^{2+}$)-ATPase activities that exhibit relatively high affinities for Ca$^{2+}$ (14, 17–21, 24, 25). However, although the Ca$^{2+}$-stimulated Mg$^{2+}$-dependent ATPase activity in synaptic membrane preparations has been studied in some detail (14, 21, 24, 25), no effort was made to determine whether there were any distinguishing characteristics between the presumed synaptic plasma membrane (Ca$^{2+}$ + Mg$^{2+}$)-ATPase activity and the enzyme activity which is associated with the membranous organelles that are isolated in the microsomal subfraction (18–20, 26). Such distinguishing characteristics have recently been reported with respect to the (Ca$^{2+}$ + Mg$^{2+}$)-ATPases of sarcosomal membranes and sarcoplasmic reticulum in heart myocytes (27). Differentiation between the properties of synaptic plasma membrane (Ca$^{2+}$ + Mg$^{2+}$)-ATPases and the microsomal membrane ATPases would be useful in determining the relative contributions of these two enzymes to neuronal Ca$^{2+}$ transport processes.

Synaptosomal membrane fractions are usually contaminated with other membranous organelles, such as mitochondria, synaptic vesicles, and endoplasmic reticulum-like structures. Thus, a rather extensive purification procedure for the isolation of synaptic plasma membranes was used in these studies to enable us to compare the subcellular distribution of enzymatic markers for the various membranous organelles with the distribution of the (Ca$^{2+}$ + Mg$^{2+}$)-ATPase in these subfractions. Subsequently, a detailed examination of the enzyme kinetics with respect to Mg$^{2+}$ dependence, Ca$^{2+}$ stimulation, and ATP dependence of the high affinity divalent cation-stimulated ATPase activities of synaptic plasma membranes was undertaken. Finally, the sensitivity of this plasma membrane enzyme to inhibition by low concentrations of vanadate was compared with that of the microsomal (Ca$^{2+}$ + Mg$^{2+}$)-ATPase as a possible means of distinguishing these two enzymes. An investigation of the presence of a distinct high affinity (Ca$^{2+}$ + Mg$^{2+}$)-ATPase enzyme in brain synaptic membranes and an exploration of the kinetic characteristics of such an enzyme system should prove helpful in efforts to elucidate the mechanisms involved in Ca$^{2+}$ disposition within nerve terminals.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Male Sprague-Dawley rats (250-350 g) were obtained from Charles River Breeding Laboratories. The following chemicals were purchased from Sigma Chemical Co.: Ficoll, Tris, adenosine monophosphate, &methyladenosine 5'-diphosphate, bovine serum albumin, Tris-ATP, CDTA, EGTA, EDTA, sodium deoxycholate, calf thymus DNA, 3-ethyl-4-methoxyacetic acid, DCCD, N,N'-dicyclohexylcarbodiimide, EGTA, ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, BSA, and sodium deoxycholate. The n-butyl acetate (spectral grade) and sodium deoxycholate were purchased from Sigma Chemical Co.: Ficoll, Tris, adenosine monophosphate, &methyladenosine 5'-diphosphate, bovine serum albumin, Tris-ATP, CDTA, EGTA, EDTA, sodium deoxycholate.

**Preparation of Synaptic Membranes and Other Subcellular Fractions**—Whole brains were rapidly removed following cervical dislocation, minced, and then homogenized in 20 volumes of cold sucrose-MgSO₄ medium in a Potter-Elvehjem glass homogenizer; with a Teflon pestle (0.13 mm clearance) by eight strokes at 1000 rpm. The preparation of synaptic membranes used in most studies of Ca²⁺-stimulated Mg²⁺-ATPase activity involved a combination of the methods described by Cotman and Matthews (28) and Salvaterra and Matthews (29) and is outlined in Fig. 1. The fraction designated as "general microsomes" was prepared according to the procedures of Black et al. (30) and it is also shown in Fig. 1. The various tissue fractions (4-6 mg of tissue protein/ml) were divided into small aliquots, frozen rapidly in liquid N₂, and stored at -80°C until used. Calcium-stimulated Mg²⁺-ATPase activity was initially tested in freshly isolated as well as rapidly frozen and thawed subcellular fractions. All the reported g forces are the maximum values for each step.

**Enzymatic Marker Assays**—The activity of the plasma membrane enzyme 5'-nucleotidase was measured as described by Edwards and Maguire (31). The specific 5'-nucleotidase activity was defined as that which was inhibited by the addition of 1 mM &methyl-adenosine 5'-diphosphate (32). The (Na⁺ + K⁺)-ATPase activity was measured in an incubation medium which contained 10-20 µg of protein, 50 mM Tris-HCl, pH 7.4, and 2 mM MgCl₂ with or without the addition of 50 mM NaCl and 10 mM KC₂ in a final volume of 1 ml. The reaction was initiated with the addition of 2 mM ATP and carried out for 20 min at 37°C, and the amount of phosphate liberated was determined as described by Yoda and Hokin (33). The distribution of the mitochondrial enzyme succinate dehydrogenase was measured by reduction of a tetrazolium salt essentially as described by Pershadsingh and al. (38) with bovine serum albumin as the standard.

**Other Procedures**—Calcium and magnesium were determined by atomic absorption spectrophotometry with a Perkin-Elmer instrument. Protein concentrations were measured by the method of Lowry et al. (35) with bovine serum albumin as the standard.

**Analysis of Data**—All kinetic data obtained from the ATPase assay were evaluated by the Eadie-Hofstee graphic procedure as described by Walter (39) since this type of graphic analysis has been shown to be the most sensitive to deviations from hyperbolic enzyme kinetics (39). The kinetic constants presented for enzymatic processes that did not have strictly hyperbolic Michaelis-Menten characteristics were obtained by fitting of the Hill equation (40).

**RESULTS**

**Synaptosomal and Synaptic Membrane (Ca²⁺ + Mg²⁺)-ATPase, Mg²⁺-ATPase, and Ca²⁺-ATPase**—Freshly prepared synaptosomes were tested within 2 h of their preparation for the activity of (Ca²⁺ + Mg²⁺)-ATPase (Fig. 2A). The addition of increasing concentrations of Ca²⁺ in the range of 0.2-20 µM enhanced the basal Mg²⁺-ATPase activity of synaptosomes, whereas concentrations of Ca²⁺ greater than 20 µM caused progressive return of the enzymatic activity toward the base-line Mg²⁺-ATPase (Fig. 2A). The results shown in Fig. 2A indicated the presence of a high affinity Ca²⁺-activated Mg²⁺-ATPase enzyme that could be stimulated by submicromolar Ca²⁺ concentrations. Since one of the major constituents of the synaptosomal particle is the synaptic plasma membrane, the presence of (Ca²⁺ + Mg²⁺)-ATPase activity in the isolated synaptic plasma membranes was also determined and is shown in Fig. 2B. As had been observed in the synaptosomes, there was an indication of a high affinity Ca²⁺-stimulated Mg²⁺-ATPase activity when Ca²⁺ concentrations in the range of 0.1-1.0 µM were tested. However, the high affinity component of the (Ca²⁺ + Mg²⁺)-ATPase measured under these conditions constituted a relatively small fraction of the total (Ca²⁺ + Mg²⁺)-ATPase activity detected at 1-100 µM Ca²⁺ concentrations. Identical levels and patterns of (Ca²⁺ + Mg²⁺)-ATPase activity were obtained in freshly prepared synaptic membranes and in membranes frozen in liquid nitrogen and stored up to 4 weeks at -80°C. Therefore, in most of the subsequent studies, the rapidly frozen membrane preparations were used.

The Ca²⁺ activation of the Mg²⁺-stimulated ATPase dif-
activities were measured in the standard incubation medium with 2 mM Mg²⁺ and 2 mM ATP as described under "Experimental Procedures." A, the results shown are from a single synapticosomal preparation and represent the mean of triplicate determinations. All assays were conducted at 37 °C and were initiated by the addition of substrate following a 5-min preincubation of the synaptosomes (57 μg/assay) in the incubation medium. B, synaptic membrane (Band 2 of sucrose gradient of Fig. 1) activity of (Ca²⁺ + Mg²⁺)-ATPase was determined under conditions identical with those described above. The membrane protein concentration in the assays varied between 19 and 21 μg. Each point is the mean activity (± S.E.) from three different preparations, each determined in triplicate.

fered from the Ca²⁺ activation of the ATPase activity in the absence of Mg²⁺, i.e. the Ca²⁺-ATPase (Fig. 3). Neither synapticosomal nor synaptic membrane Ca²⁺-ATPase activities exhibited any inhibition at Ca²⁺ concentrations ranging from 0.1–2 mM, and they exhibited Kₐ₅ values for Ca²⁺ of 0.16 and 0.25 mM, respectively (Fig. 3). These Ca²⁺-ATPases had Kₐ₅ values similar to those of the Mg²⁺-dependent ATPases of synaptosomes and synaptic membranes (Kₐ₅ for Mg²⁺ = 0.28 mM for synaptosomes; Kₐ₅ = 0.31 mM for synaptic membranes), but they had approximately half the maximal activities, as can be seen in Fig. 3. Divalent cation concentrations greater than 2 mM, i.e. in excess of the 2 mM ATP substrate concentration, caused a small degree of inhibition of enzyme activity (Fig. 3). Optimal activation was obtained with either 2 mM Mg²⁺ or 2 mM Ca²⁺. The Kₐ₅ values for Ca²⁺ and Mg²⁺ indicated above are for total divalent cation concentrations. If one calculates the Kₐ₅ values using the free Ca²⁺ or Mg²⁺ concentrations as described by Pershad Singh and McDonald (37), the following values are obtained: Kₐ₅ for free Ca²⁺ = 19.5 μM for the synaptosomes and 39 μM for the synaptic membranes, and Kₐ₅ for free Mg²⁺ = 37 and 44 μM for these preparations, respectively.

Since the Ca²⁺ concentrations that enhanced the high affinity component of the (Ca²⁺ + Mg²⁺)-ATPase were in the submicromolar range, it seemed necessary to determine whether the membranes brought a sufficient amount of Ca²⁺ to contribute to the final Ca²⁺ concentration in the assay and to the stimulation of the basal Mg²⁺-ATPase. The amounts of Ca²⁺ and Mg²⁺ associated with the synaptic membrane preparations were determined by atomic absorption spectrophotometry. The membrane contribution to the [Ca²⁺] was less than 40 nM and that to the [Mg²⁺] was 0.5 nM or less. In addition, the Ca²⁺-induced stimulation of the Mg²⁺-ATPase in membranes that had been pretreated with 0.1 mM EGTA for 7 min at 23 °C was very similar to that measured in membranes not treated with EGTA (data not shown). Somewhat higher Ca²⁺ concentrations (1–10 μM) were needed to stimulate the high affinity component of this enzyme in the EGTA-treated membranes.

The high affinity Ca²⁺-stimulated Mg²⁺-ATPase of the synaptic plasma membranes was studied in greater detail through the use of CDTA-buffered Ca²⁺ and Mg²⁺ incubation media in order to control more precisely the Ca²⁺ and Mg²⁺ concentrations at the micromolar and submicromolar levels (37). No basal Mg²⁺-ATPase or Ca²⁺-stimulated Mg²⁺-ATPase activity was detectable in these preparations in the absence of added Mg²⁺ or when only submicromolar free Ca²⁺ concentrations were tested without Mg²⁺ addition. The Mg²⁺-ATPase in the synaptic plasma membranes had a lower Kₐ₅ for Mg²⁺ when the Mg²⁺-induced activation of the enzyme was measured in the CDTA buffer medium as compared with a medium containing no CDTA (Fig. 4A versus Fig. 3B). The Kₐ₅ for Mg²⁺ in CDTA-containing medium was 6.6 μM free Mg²⁺ (Fig. 4A), whereas that determined in the absence of a chelator was 44 μM free Mg²⁺ (Fig. 3). The Kₐ₅ for Mg²⁺ enhancement of the ATPase activity in the CDTA buffer medium was not substantially altered (Kₐ₅ = 6.0 μM) by the presence of 1.0 μM Ca²⁺ in the incubation medium (Fig. 4A). However, introduction of 1.0 μM Ca²⁺ produced a substantial increase in the Vₐ₅ of the Mg²⁺-ATPase activity, from 6.17 to 8.58 μmol/mg/h (Fig. 4A).

Stimulation of the basal Mg²⁺-ATPase of synaptic plasma membranes was dependent on the concentration of Ca²⁺ in the assay medium (Fig. 4B). The Kₐ₅ for Ca²⁺ stimulation of the Mg²⁺-ATPase was estimated to be 0.23 μM, definitely within the concentration range of the previously detected high affinity Ca²⁺-stimulated Mg²⁺-ATPase of synaptic plasma membranes (Fig. 2).

When ATPase activity was measured in the CDTA buffer medium in the presence of 5 μM Mg²⁺, the enzyme also exhibited high affinity for the substrate ATP (Fig. 4C). The basal Mg²⁺-ATPase activity had a Kₐ₅ of 23.8 μM for ATP, and the Ca²⁺-stimulated Mg²⁺-ATPase had a Kₐ₅ of 18.9 μM (Fig. 4C). These estimates of the Kₐ₅ for the enzyme were obtained from the linear segments of Eadie-Hofstee plots (Fig. 4C, inset), which also showed the presence of apparent positive cooperativity in the activation of these ATPases by ATP. A Hill coefficient of 1.9 and Kₐ₅ of 26.5 μM were calculated from the Hill equation. The corresponding constants for the Ca²⁺-stimulated Mg²⁺-ATPase in these membranes were a Hill coefficient of 1.5 and Kₐ₅ of 20.4 μM ATP. Although the presence of 1 μM Ca²⁺ did not significantly alter the Kₐ₅ of the enzyme for ATP, it increased the Vₐ₅ as determined from the Eadie-Hofstee plots from 1.76 μmol/mg/h for the basal Mg²⁺-ATPase to 2.38 μmol/mg/h for the Ca²⁺-stimulated Mg²⁺-ATPase (Fig. 4C, inset). All assays were conducted within the linear phase of product formation by
varying the incubation periods, especially for the samples that contained ATP in the range of 5–50 μM.

Thus, the characteristics of the high affinity synaptic plasma membrane (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase were found to be: (a) strict dependence on the presence of Mg\textsuperscript{2+} for the appearance of the ATPase activity; (b) high affinity for Ca\textsuperscript{2+} (K\textsubscript{M} = 0.23 μM); (c) relatively high affinity for both Mg\textsuperscript{2+} and ATP (K\textsubscript{M} = 6.6 μM for Mg\textsuperscript{2+} and K\textsubscript{M} = 18.9 μM for ATP); and (d) positive cooperativity in the activation of the enzyme by ATP (Hill coefficient = 1.5). In addition to these general characteristics, the (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase activity in these membranes was not inhibited either by ouabain or by oligomycin nor was it significantly affected by changes in the concentrations of K\textsuperscript{+} or Na\textsuperscript{+} (0–80 mM) in the incubation medium. A small degree of enhancement of (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase activity was observed in the presence of 25–50 mM K\textsuperscript{+} (10–15%) and none was detected in the presence of varying concentrations of Na\textsuperscript{+} (data not shown).

Subcellular Distribution of High Affinity (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase in Rat Brain—The subcellular distribution of the high affinity (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase was compared with that of other enzymatic markers for mitochondria (succinate dehydrogenase), microsomes (glucose-6-phosphatase), and plasma membranes (5’-nucleotidase and (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase) as shown in Table I. The purified synaptic membranes contained 2.0% of the succinate dehydrogenase, 2.6% of the protein, none of the glucose-6-phosphatase, 7.1% of the (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase, and 12.2% of the 5’-nucleotidase activity associated with the crude mitochondrial (P2) fraction. On the basis of the distribution of these enzymatic markers, it was apparent that the synaptic membrane fraction was enriched with plasma membrane particles. There was some contamination with mitochondrial enzymatic activity despite the fact that electron microscopic examination of this membrane fraction revealed the presence of very few recognizable mitochondrial particles. It is possible that the succinate dehydrogenase activity represents contamination with mitochondrial membrane fragments rather than with intact mitochondria. The pellet from the sucrose density gradient (P3) did contain predominantly intact mitochondria and lysed synaptosomes as determined by electron microscopic examination. In addition, 21.2% of the succinate dehydrogenase activity of the crude mitochondrial fraction was associated with this pellet, while only 9.6% of the protein, 2.3% of the 5’-nucleotidase, and 6.9% of the protein, 2.3% of the 5’-nucleotidase, and 6.9% of the 5’-nucleotidase activity associated with the crude mitochondrial (P2) fraction. On the basis of the distribution of these enzymatic markers, it was apparent that the synaptic membrane fraction was enriched with plasma membrane particles.
with the pellet from the sucrose density gradients (P₄) was considered to be due to contaminating subcellular particles such as intact synaptosomes found in this subfraction. Since the (Ca²⁺ + Mg²⁺)-ATPase activity of the microsomes was the only one comparable to that of the synaptic membranes, the microsomal enzyme was studied in greater detail under the conditions used for investigating the synaptic membrane enzyme.

Microsomal (Ca²⁺ + Mg²⁺)-ATPase—There was relatively little stimulation of the basal Mg²⁺-ATPase activity in microsomes by Ca²⁺ concentrations ranging from 0.1-5 μM (Fig. 5A). Treatment of the microsomes with 0.1% (w/v) Na deoxycholate for 2.5 min at 23 °C resulted in a substantial enhancement of the Ca²⁺-induced activation of the basal Mg²⁺-ATPase and in the appearance of a high affinity component of this enzyme sensitive to the presence of submicromolar concentrations of Ca²⁺ (Fig. 5A). It has been observed that deoxycholate treatment (0.9%, w/v) led to an increased sensitivity to Ca²⁺ in the adipocyte microsomal Mg²⁺-ATPase (30) and Lubrol WX treatment had a similar effect in brain microsomes (26). However, unlike the adipocyte microsomal enzyme, the brain microsomal Mg²⁺-ATPase activity did not decrease significantly following treatment of the membranes with 0.1% (w/v) deoxycholate (Fig. 5A).

The brain microsomal (Ca²⁺ + Mg²⁺)-ATPase activity was also examined in the presence of 5 μM Mg²⁺, 1 mM ATP, and the CDTA ion-buffering system. Enzymatic activity with high affinity for Ca²⁺ stimulation was also observed under these conditions (Fig. 5B). The estimated Kₐ₅ for Ca²⁺ was 0.48 μM. Under both sets of conditions used to measure the activity of the (Ca²⁺ + Mg²⁺)-ATPase, the high affinity Ca²⁺-stimulated Mg²⁺-ATPase represented a smaller fraction of the total ATPase activity in the microsomes than it did in the synaptic membranes.

Inhibition of Synaptic and Microsomal Membrane (Ca²⁺ + Mg²⁺)-ATPase by Vanadate—It has recently been suggested that the differential sensitivity of plasma membrane and sarcoplasmic reticulum (Ca²⁺ + Mg²⁺)-ATPase to the inhibitory effects of vanadate at concentrations below 10 μM can be used to distinguish the Ca²⁺-activated enzyme of the plasma membranes from that of the sarcoplasmic reticulum in cardiac tissue (27). If the brain microsomal enzyme were similar in its characteristics to the sarcoplasmic reticulum enzyme, then one might expect to see a relative insensitivity of this enzyme to inhibition by vanadate at concentrations below 10 μM. When the brain microsomal and synaptic plasma membranes were incubated in the presence of 0.25-50 μM vanadate, only the synaptic plasma membrane (Ca²⁺ + Mg²⁺)-ATPase activity was significantly inhibited by vanadate concentrations below 10 μM (Fig. 6A). Both types of (Ca²⁺ + Mg²⁺)-ATPase activity were sensitive to the higher (10-50 μM) vanadate concentration (Fig. 6). Half-maximal inhibition of the synaptic membrane (Ca²⁺ + Mg²⁺)-ATPase was produced by 2.7 μM vanadate. The maximum inhibition achieved with concentrations as high as 100 μM was 74% inhibition of the (Ca²⁺ + Mg²⁺)-ATPase; the high affinity Ca²⁺-stimulated Mg²⁺-ATPase activity was only 20% inhibited by 100 μM vanadate (Fig. 6B).
**Activity**—The synaptic plasma membrane (Ca\textsuperscript{2+}-ATPase, as well as on the electron microscopic detection of orthovanadate. Assays were performed as described in Fig. 4. Each point is the mean activity (± S.E.) from four membrane preparations measured in triplicate.

**Table II**

Effects of various inhibitors on the (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase and on the Mg\textsuperscript{2+}-ATPase of synaptic membranes

<table>
<thead>
<tr>
<th>Agent concentration (µM)</th>
<th>Mg\textsuperscript{2+}-ATPase</th>
<th>Inhibition (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase</th>
<th>Inhibition Mg\textsuperscript{2+}-ATPase %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.55 ± 0.19</td>
<td>1.72 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Lanthanum</td>
<td>5.08</td>
<td>5.08</td>
<td>82.6</td>
</tr>
<tr>
<td>25</td>
<td>5.18</td>
<td>5.18</td>
<td>82.6</td>
</tr>
<tr>
<td>DCCD</td>
<td>1.75 ± 0.24</td>
<td>68.5</td>
<td>43.0</td>
</tr>
<tr>
<td>100</td>
<td>0.46 ± 0.08</td>
<td>73.3</td>
<td>70.3</td>
</tr>
</tbody>
</table>

**DISCUSSION**

High affinity Ca\textsuperscript{2+}-stimulated Mg\textsuperscript{2+}-dependent ATPase activity was not significantly inhibited by 0.25–50 µM vanadate while that of the microsomal membranes was inhibited to a small extent (4–9%).

**Effects of La\textsuperscript{3+} and DCCD on Synaptic Membrane ATPase Activity**—The synaptic plasma membrane (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase was strongly inhibited by 25 and 50 µM La\textsuperscript{3+}, whereas the basal Mg\textsuperscript{2+}-ATPase activity of these membranes was essentially unaffected by La\textsuperscript{3+} (Table II). On the other hand, DCCD inhibited both the Mg\textsuperscript{2+}-ATPase and the (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase activities of the synaptic membranes to a nearly equivalent extent (Table II).

**Vanadate inhibition of synaptic membrane** (A) and microsomal membrane (B) high affinity (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase. The membranes were preincubated in CDTA-buffered medium in the presence of 5 µM Mg\textsuperscript{2+}, 1 mM Ca\textsuperscript{2+}, and varying concentrations of orthovanadate. Assays were performed as described in Fig. 4. Each point is the mean activity (± S.E.) from four membrane preparations measured in triplicate.
observed (46) that the use of EGTA to buffer Ca\(^{2+}\) concentrations in the assay of erythrocyte membrane (Ca\(^{2+} + \text{Mg}^{2+}\))-ATPase decreased the K\(_{0.5}\) for Ca\(^{2+}\) activation of the enzyme as compared to the values estimated in the absence of EGTA. The magnitude of the decrease in the K\(_{0.5}\) for Ca\(^{2+}\) activation in the presence of EGTA was quite similar to that observed in the K\(_{0.5}\) for Mg\(^{2+}\) activation of the enzymes of synaptic membranes in the CDTA-buffered medium.

The results presented in this study indicate that both the microsomal membranes and the synaptic plasma membranes have (Ca\(^{2+} + \text{Mg}^{2+}\))-ATPase activities and are likely to be involved in ATP-dependent Ca\(^{2+}\) transport processes in brain neurons. It is quite possible that one of the primary ATP-dependent Ca\(^{2+}\)-transporting systems for maintaining Ca\(^{2+}\) homeostasis within nerve terminals is an extrusion of Ca\(^{2+}\) across the synaptic plasma membrane. However, the demonstration of the presence of high affinity (Ca\(^{2+} + \text{Mg}^{2+}\))-ATPase activity in synaptic plasma membranes and the detection of an ATP-dependent Ca\(^{2+}\) transport in these isolated membrane vesicles would not be sufficient to establish the existence of a Ca\(^{2+}\) extrusion process across neuronal membranes which operates via a (Ca\(^{2+} + \text{Mg}^{2+}\))-ATPase system. In order to reach such a conclusion, the orientation of the (Ca\(^{2+} + \text{Mg}^{2+}\))-ATPase enzymes in these membranes and the directionality of Ca\(^{2+}\) transport across the synaptic plasma membrane will have to be determined.

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