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

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High affinity Ca\textsuperscript{2+}-stimulated Mg\textsuperscript{2+}-dependent ATPase activity of nerve ending particles (synaptosomes) from rat brain tissue appears to be associated primarily with isolated synaptic plasma membranes. The synaptic membrane (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase activity was found to exhibit strict dependence on Mg\textsuperscript{2+} for the presence of the activity, a high affinity for Ca\textsuperscript{2+} (K\textsubscript{D} = 0.23 \mu M), and relatively high affinities for both Mg\textsuperscript{2+} and ATP (K\textsubscript{D} = 6.0 \mu M for Mg\textsuperscript{2+} and K\textsubscript{M} = 18.9 \mu M for ATP). These kinetic constants were determined in incubation media that were buffered with the divalent cation chelator trans-cyclohexane-1,2-diamine-N\textsubscript{2}N\textsubscript{2}N\textsubscript{2}N\textsuperscript{-tetaacetic acid. The enzyme activity was not inhibited by ouabain or oligomycin but was sensitive to low concentrations of vanadate. The microsomal membrane subfraction was the other brain subcellular fraction with a high affinity (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase activity which approximated that of the synaptic plasma membranes. The two membrane-related high affinity (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase activities could be distinguished on the basis of their differential sensitivity to vanadate at concentrations below 10 \mu M. Only the synaptic plasma membrane (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase was inhibited by 0.25-10 \mu M vanadate. The studies described here indicate a possible involvement of both the microsomal and the neuronal plasma membrane (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase in high affinity Ca\textsuperscript{2+} transport across membranes of brain neurons. In addition, they suggest a means by which the relative contributions of each transport system might be evaluated based on their differential sensitivity to inhibition by vanadate.

The regulation of intraneuronal free calcium ion concentrations, [Ca\textsuperscript{2+}]\textsubscript{i}, within the range of 0.1-1.0 \mu M 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\textsuperscript{2+}]\textsubscript{i} include the transport of Ca\textsuperscript{2+} into mitochondria (5, 6), the binding of Ca\textsuperscript{2+} by intraneuronal or membrane proteins (7, 8), the exchange of intraneuronal Ca\textsuperscript{2+} with extracellular Na\textsuperscript{+} through a Na\textsuperscript{+}:Ca\textsuperscript{2+} antiport process (9-11), and finally, Ca\textsuperscript{2+} extrusion or sequestration by Ca\textsuperscript{2+}-activated ATPases (5, 12-15).

The ATP-dependent transport of Ca\textsuperscript{2+} has been viewed by some investigators as a sequestration of Ca\textsuperscript{2+} within vesicular structures present in nerve terminals (5, 13, 22, 23). It has been proposed by others, however, that ATP-dependent Ca\textsuperscript{2+} transport in nerve terminal preparations (synaptosomal membranes) is a plasma membrane process that leads to Ca\textsuperscript{2+} extrusion from the intra- to the extraneuronal space (14, 18, 19, 21, 24). The Ca\textsuperscript{2+}-stimulated Mg\textsuperscript{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\textsuperscript{2+} transport. Both synaptosomal membrane preparations and microsomal membranes have been shown to have (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase activities that exhibit relatively high affinities for Ca\textsuperscript{2+} (14, 17-21, 24, 25). However, although the Ca\textsuperscript{2+}-stimulated Mg\textsuperscript{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\textsuperscript{2+} + Mg\textsuperscript{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\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPases of sarcolemmal membranes and sarcoplasmic reticulum in heart myocytes (27). Differentiation between the properties of synaptic membrane (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPases and those of the microsomal membranes would be useful in determining the relative contributions of these two enzymes to neuronal Ca\textsuperscript{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\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase in these subfractions. Subsequently, a detailed examination of the enzyme kinetics with respect to Mg\textsuperscript{2+} dependence, Ca\textsuperscript{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\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase as a possible means of distinguishing these two enzymes. An investigation of the presence of a distinct high affinity (Ca\textsuperscript{2+} + Mg\textsuperscript{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\textsuperscript{2+} disposition within nerve terminals.

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Rat Brain ATPase Activity

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, α,β-methylene adenosine 5'-diphosphate, bovine serum albumin, Tris-ATP, CDTA, EGTA, EDTA, sodium deoxycholate, ouabain, oligonuclease, DCCD, indomethacin, sodium azide, and sodium succinate. The n-butyl acetate (spectral grade) and sodium deoxycholate were purchased from Sigma Chemical Co. Fisher Scientific was the source for the other reagents used and all were of reagent grade. The distilled water used in preparation of all solutions was deionized in a multichambered mixed bed ion exchange resin system.

Preparation of Brain 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 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 α,β-methylene 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 KCl, in a final volume of 1 ml. The reaction was initiated with the addition of 2 mM ATP to give a final concentration of 20 mM, 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 Green and Narahara (34), but using extraction with ethyl acetate and phosphomolybdate complex was extracted into butyl acetate as described by Yoda and Hokin (33). The activity of the microsomal enzyme glucose-6-phosphatase was measured according to the procedures of Nordlie and Arion (36).

**ATPase Assays**—The activity of Mg²⁺-ATPase, Ca²⁺-ATPase and the Ca²⁺-stimulated Mg²⁺-ATPase was measured with or without the CDTA buffer depending upon the need to control the free Ca²⁺ and Mg²⁺ concentrations in the submicromolar range. The assay system contained 12–20 μg of tissue protein, 50 mM KCl, 25 mM Tris-HCl, pH 7.4, 1 or 2 mM ATP, as indicated, 4 μg of oligomycin, 0.1 mM ouabain, in a final volume of 1 ml with the [MgCl₂], [CaCl₂], and 0.2 mM CDTA present as indicated for individual figures. The reaction was initiated by the addition of ATP, allowed to proceed for 20 min (unless indicated otherwise) 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 Green and Narahara (34), but using extraction with ethyl acetate and phosphomolybdate complex was extracted into butyl acetate as described by Yoda and Hokin (33). The activity of the microsomal enzyme glucose-6-phosphatase was measured according to the procedures of Nordlie and Arion (36).

**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. (38) with bovine serum albumin as the standard.

**Analysis of Data**—All kinetic data obtained from the ATPase assay were analyzed 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 were determined as described by Michaelis and Menten (41). The Km's were determined from the Michaelis-Menten equation and the Vmax. was determined from the Eadie-Hofstee equation.

**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 basal Mg²⁺-ATPase activity (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 mM 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 Mg" and 2 mM ATP as described under "Experimental Procedures." A, the results shown are from a single synaptosomal 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 through the use of CDTA-buffered Ca' and Mg" incubation medium in order to control more precisely the Ca' and Mg' concentrations at the micromolar and submicromolar levels (37). No basal Mg"-ATPase or Ca' -stimulated Mg"-ATPase activity was detectable in these preparations in the absence of added Mg" or when only submicromolar free Ca" concentrations were tested without Mg" addition. The Mg"-ATPase in the synaptic plasma membranes had a lower K0.5 for Mg" when the Mg"-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 K0.5 for Mg" in CDTA-containing medium was 6.6 μM free Mg" (Fig. 4A), whereas that determined in the absence of a chelator was 44 μM free Mg" (Fig. 3). The K0.5 for Mg" enhancement of the ATPase activity in the CDTA buffer medium was not substantially altered (K0.5 = 6.0 μM) by the presence of 1.0 μM Ca" in the incubation medium (Fig. 4A). However, introduction of 1.0 μM Ca" produced a substantial increase in the Vmax of the Mg"-ATPase activity, from 6.17 to 8.58 μmol/mg/h (Fig. 4A).

Stimulation of the basal Mg"-ATPase of synaptic plasma membranes was dependent on the concentration of Ca" in the assay medium (Fig. 4B). The K0.5 for Ca" stimulation of the Mg"-ATPase was estimated to be 0.23 μM, definitely within the concentration range of the previously detected high affinity Ca"-stimulated Mg"-ATPase of synaptic plasma membranes (Fig. 2).

When ATPase activity was measured in the CDTA buffer medium in the presence of 5 μM Mg", the enzyme also exhibited high affinity for the substrate ATP (Fig. 4C). The basal Mg"-ATPase activity had a K0.5 of 23.8 μM for ATP, and the Ca"-stimulated Mg"-ATPase had a K0.5 of 18.9 μM (Fig. 4C). These estimates of the K0.5 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 K0.5 of 26.5 μM were calculated from the Hill equation. The corresponding constants for the Ca"-stimulated Mg"-ATPase in these membranes were a Hill coefficient of 1.5 and K0.5 of 20.4 μM ATP. Although the presence of 1 μM Ca" did not significantly alter the K0.5 of the enzyme for ATP, it increased the Vmax as determined from the Eadie-Hofstee plots from 1.76 μmol/mg/h for the basal Mg"-ATPase to 2.38 μmol/mg/h for the Ca"-stimulated Mg"-ATPase (Fig. 4C, inset). All assays were conducted within the linear phase of product formation by...
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The characteristics of the high affinity synaptic plasma membrane (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase were found to be: (a) strict dependence on the presence of Mg\(^{2+}\) for the appearance of the ATPase activity; (b) high affinity for Ca\(^{2+}\) (K\(_D\) = 0.23 \(\mu\)M); (c) relatively high affinity for both Mg\(^{2+}\) and ATP (K\(_D\) = 6.5 \(\mu\)M for Mg\(^{2+}\) and K\(_M\) = 18.9 \(\mu\)M 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\(^{2+}\) + Mg\(^{2+}\))-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\(^+\) or Na\(^+\) (0-80 \(\mu\)M) in the incubation medium. A small degree of enhancement of (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity was observed in the presence of 26-50 \(\mu\)M K\(^+\) (10-15\%) and none was detected in the presence of varying concentrations of Na\(^+\) (data not shown).

Subcellular Distribution of High Affinity (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase in Rat Brain—The subcellular distribution of the high affinity (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase was compared with that of other enzymatic markers for mitochondria (succinate dehydrogenase), microsomes (glucose-6-phosphatase), and plasma membranes (5'-nucleotidase and (Na\(^+\) + K\(^+\))-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\(^+\) + K\(^+\))-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 (P6) did contain predominantly intact mitochondria and unlysed synapticosomes 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 (Na\(^+\) + K\(^+\))-ATPase were recovered in this fraction (Table I).

The (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity that was measured following osmotic lysis and multiple centrifugations of the crude mitochondrial and synaptosomal fractions appeared to be somewhat labile, with greater losses in specific activity observed with more prolonged handling of the various fractions. Nevertheless, of the various subfractions obtained following osmotic lysis of the synaptosomes, the highest specific activity of the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase was associated with the isolated synaptic plasma membrane fraction. However, the net Ca\(^{2+}\)-stimulated Mg\(^{2+}\)-ATPase activity of the "general microsomal" fraction (P6) was comparable to that of the synaptic membranes (Table I). The membranous fraction that contained intrasynaptosomal vesicles and microsomes (P1 in Fig. 1) exhibited low (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity under these assay conditions (Table I), and the myelin-containing fraction from the 10-28.5\% sucrose interface had low or no detectable (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity (data not shown). The Ca\(^{2+}\)-stimulated Mg\(^{2+}\)-ATPase associated

![Graphs showing activation of synaptic plasma membrane Mg\(^{2+}\)-ATPase and high affinity (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase by Mg\(^{2+}\), Ca\(^{2+}\), and ATP.](image)

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that the differential sensitivity of plasma membrane and used to distinguish the Ca2+-activated enzyme of the plasma
tory effects of vanadate at concentrations below 10
M$+-ATPase activity in the microsomes than it did in the synaptic
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 submicro-
lar 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 K0.5 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 syn-
aptic membrane (Ca2+ + Mg2+)-ATPase was produced by 2.7 μM vanadate. The maximum inhibition achieved with concen-
trations as high as 100 μM was 74% inhibition of the (Ca2+ +
Mg\(^{2+}\))-ATPase. The synaptic membrane basal Mg\(^{2+}\)-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\(^{3+}\) and DCCD on Synaptic Membrane ATPase Activity**—The synaptic plasma membrane (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase was strongly inhibited by 25 and 50 µM La\(^{3+}\), whereas the basal Mg\(^{2+}\)-ATPase activity of these membranes was essentially unaffected by La\(^{3+}\) (Table II). On the other hand, DCCD inhibited both the Mg\(^{2+}\)-ATPase and the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activities of the synaptic membranes to a nearly equivalent extent (Table II).

**DISCUSSION**

High affinity Ca\(^{2+}\)-stimulated Mg\(^{2+}\)-dependent ATPase activity measured in intact nerve endings (synaptosomes) from rat brain tissue has been shown to be primarily associated with the plasma membranes of these neuronal preparations. Intrasympotosomal vesicular structures had low activity of this (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase, and the enzyme activity measured in the intrasympotosomal mitochondrial subfraction appeared to be due to contamination of this subfraction with unruptured or partially ruptured synaptosomes. The latter conclusion was based on the distribution of the plasma membrane enzymatic markers 5'-nucleotidase and (Na\(^{+}\) + K\(^{+}\))-ATPase, as well as on the electron microscopic detection of recognizable synaptosomal structures in the fraction that contained intrasympotosomal mitochondria.

The only other subcellular fraction which exhibited (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity nearly equivalent to that of the synaptic plasma membranes was the microsomal membrane fraction. However, the distribution of the microsomal enzyme glucose-6-phosphatase indicated that a clear separation between synaptic plasma membranes and microsomes had been achieved. Although the precise cellular origin of the membranes in the microsomal fraction is not known, it appears that they are partially derived from neuronal or glial endoplasmic reticulum membranes as indicated by the high glucose-6-phosphatase activity in this fraction (41). There was a relatively small difference between the K\(_{50}\) for Ca\(^{2+}\)-induced stimulation of the basal Mg\(^{2+}\)-ATPase in synaptic plasma membranes and in microsomal membranes (K\(_{50}\) = 0.23 and 0.48 µM for the synaptic membranes and the microsomes, respectively). However, the microsomal (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase was insensitive to inhibition by low concentrations of vanadate (<10 µM) whereas the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase of the synaptic plasma membranes was quite sensitive to vanadate in this concentration range. Thus, in terms of sensitivity to low concentrations of vanadate, the activity of the synaptic plasma membrane (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase is similar to that of erythrocyte and sarcolemmal plasma membranes, while that of brain microsomal membranes is similar to the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase of the sarcoplasmic reticulum (27). It has previously been reported that 1 µM vanadate caused 25% or 30% inhibition of the brain microsomal (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase depending on whether KC1 was present (19). No explanation can be provided for the differences in the results obtained in this study and those reported in the present study, particularly since all of our (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase assays with microsomal membranes were conducted in the presence of 50 mM KC1.

The similarity between the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase of other cell membranes and the synaptic plasma membrane (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase was apparent also in the relatively high sensitivity of the synaptic membrane enzyme to the inhibitory effects of La\(^{3+}\). Such La\(^{3+}\)-induced inhibition of (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity has been observed previously with erythrocyte, adipocyte, and synaptic membrane preparations (21, 37, 42, 43). Lanthanum did not inhibit the brain divalent cation ATPase activity of the synaptic membranes in our studies or those previously reported (21, 43), and thus it was a specific inhibitor of the Ca\(^{2+}\)-stimulated, Mg\(^{2+}\)-dependent ATPase. On the other hand, DCCD had a strong inhibitory effect on both the basal Mg\(^{2+}\)-ATPase and the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase. This reagent is known to inhibit a variety of ion transport ATPases including the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPases of neuronal and other membrane preparations (21, 44, 45).

Previously reported kinetic properties of various (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPases and ATP- and Mg\(^{2+}\)-dependent Ca\(^{2+}\) transport processes in neuronal membranes are summarized in Table III and are compared to the values obtained in the present study. The estimated kinetic constants for the synaptic membrane (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase obtained in this study are approximately equal to some of the constants for this enzymatic activity in synaptic and axonal membranes and for the ATP and Mg\(^{2+}\)-dependent Ca\(^{2+}\) transport in synaptosomal membranes. The K\(_{50}\) for Mg\(^{2+}\) for both the Mg\(^{2+}\)-ATPase as well as for the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase of synaptic membranes reported in the present study was 6.6 and 6.0 µM, respectively, a lower value than the estimated K\(_{50}\) for Mg\(^{2+}\) in the squid axonal membrane (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase (Table III). The lower K\(_{50}\) for Mg\(^{2+}\) in our studies is apparently due to the presence of the CDTA medium, since in the absence of CDTA the estimated K\(_{50}\) values for (free) Mg\(^{2+}\) activation of the basal Mg\(^{2+}\)-ATPases were 44 µM for the synaptic membranes and 37 µM for the synaptosomes. It has previously been
observed (46) that the use of EGTA to buffer Ca\(^{2+}\) concentrations in the assay of erythrocyte membrane (Ca\(^{2+}\) + 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\(^{2+}\) + 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\(^{2+}\) + 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\(^{2+}\) + Mg\(^{2+}\))-ATPase system. In order to reach such a conclusion, the orientation of the (Ca\(^{2+}\) + 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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