Calcium Transport Mechanisms in Membrane Vesicles from Guinea Pig Brain Synaptosomes*

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Ca2+ transport mechanisms were investigated using membrane vesicles prepared from guinea pig brain synaptosomes by hypotonic lysis. Two major mechanisms of Ca2+ transport exist, Na+-Ca2+ exchange and ATP-dependent Ca2+ uptake. A third although minor component, VO43- uptake occurs under hyperpolarizing conditions (determined by increased uptake of [3H]tetrphenylphosphonium+). Na+-Ca2+ exchange results in a rapid increase of [Ca2+]i (up to 100-fold above [Ca2+]o), has a Km for Ca2+ of 40 μM, is fully reversed by added external Na+, is inhibited by agents dissipating Na+ gradients (monensin or veratridine), and is uninfluenced by mitochondrial inhibitors. ATP-dependent Ca2+ uptake has a higher affinity for Ca2+ (Km = 12 μM), is dependent on Mg2+ or Mn2+, and is inhibited by β,γ-imidoadenosine 5'-triphosphate and VO43-, although only slightly (20%) inhibited by high concentrations of mitochondrial inhibitors. Both mechanisms are temperature-dependent, fully reversed by A23187, and higher in the presence of external K+. Ca2+ loaded in vesicles via ATP-dependent Ca2+ uptake is rapidly effluxed upon addition of external Na+ (as for Na+-Ca2+ exchange). Therefore a single population of vesicles exists containing both Ca2+ transport mechanisms. The two mechanisms are independent since they accumulate Ca2+ additively, are selectively inhibited by monensin and VO43-, and show distinct specificity toward other divalent cations and La3+. Although independent, Na+ (100 mM) inhibits ATP-dependent Ca2+ uptake (Km for ATP increased from 40 to 300 μM) in the absence of any net Na+ movement. Since Na+-Ca2+ exchange functions in the synaptosomal plasma membrane, the results suggest that both Ca2+ transport mechanisms originate from this membrane and function in the present experiments in inverted plasma membrane vesicles.

The regulation of calcium concentrations within nerve terminals is known to be an important controlling mechanism in the coupling of excitation with ultimate release of neurotransmitter into the synaptic space (reviewed in Ref. 1). The essential role of calcium in this process was first demonstrated by Katz and Miledi (2), and since then the mechanisms that precisely control levels of cytoplasmic calcium have been studied extensively. The concentration of free Ca2+ inside resting nerve cells (approximately 0.1 μM) is maintained against an extracellular concentration approximately 10,000-fold higher by Ca2+ transport mechanisms either directly or indirectly linked to energy-dependent ATPases.

The regulation of Ca2+ influx and efflux in squid and similar large axons has been well characterized (recently reviewed in Refs. 3 and 4) since it is possible to directly control and measure both the internal and external ionic composition. Evidence suggests that depolarization of the resting membrane potential causes an influx of Ca2+ into the axon (1, 3, 4). Extrusion of Ca2+ from the squid axon against its concentration gradient is at least partly accomplished through a mechanism within the plasma membrane whereby external Na+ entering the cell exchanges with intracellular Ca2+ via a mediated antiport system referred to as Na+-Ca2+ exchange (1, 3, 4). Recently, an ATP-dependent Ca2+ extrusion mechanism functionally resembling the cytosolic-facing (Ca2+ + Mg2+)-ATPase of red blood cell plasma membranes has been observed in the squid axon (5, 6). Thus the axon plasma membrane contains two independent Ca2+ efflux mechanisms—Na+-Ca2+ exchange and ATP-dependent Ca2+ uptake.

In contrast with the squid axon, direct measurement of Ca2+ movements in mammalian nerve terminals cannot easily be undertaken. Instead, Ca2+ fluxes have been measured indirectly using synaptosomes (pinched off, resealed presynaptic nerve terminals) as originally prepared by Gray and Whittaker (7), or subcellular fractions derived from them. As in the squid axon, evidence suggests that conditions depolarizing the resting membrane potential (interior negative) of synaptosomes, for example, high external K+ or increased Na+ permeability due to added veratridine (8), also increase the influx of Ca2+ and release of neurotransmitter (9). Influx (10, 11) and efflux (11, 12) of Ca2+ by intact synaptosomes are, respectively, inhibited and enhanced by external Na+ such that a Na+-Ca2+ exchange mechanism similar to the squid axon also operates in the synaptosomal plasma membrane (10-12). Various nonmitochondrial, subcellular fractions from brain tissue contain directly coupled, energy-dependent mechanisms serving to transport Ca2+. Thus ATP enhances the accumulation of Ca2+ into lysed synaptosomal particulate suspensions (13-15), microsomal fractions (16-18), and intraterminal neurotransmitter storage vesicles (19, 20). Such uptake processes appear to function through one or more specific (Ca2++ Mg2+)-ATPases shown to exist in corresponding fractions from the brain (17-19, 21-23). Due to heterogeneity within these fractions, the true location of (Ca2++ Mg2+)-ATPase activity in the synaptosome is questionable, although, by analogy with several other tissues, it has been suggested that endoplasmic reticulum may enzymatically accumulate Ca2+ in the brain (13-15).

In the present report, evidence is described to suggest that a single population of vesicular membranes derived from synaptosomes contains both reversible Na+-Ca2+ exchange and ATP-dependent Ca2+ uptake processes, each utilizing separate mechanisms with distinct characteristics of inhibition.

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and ionic specificity. Evidence further suggests that these vesicles may be inverted sacs of the synaptosomal plasma membrane, analogous in function with the squid axon plasma membrane described above.

**EXPERIMENTAL PROCEDURES**

**Preparation of Synaptosomal Membrane Vesicles**—A preparation of membrane vesicles was derived from guinea pig synaptosomes by a method similar to that of Kanner (24). Isolation of synaptosomes from cerebral cortex was similar to a modification (25) based on the original preparation of Gray and Whittaker (7). Brains from 10 female, 3-month-old guinea pigs killed by an overdose of urethane were removed and slices of cortex were placed in mannitol-EDTA (0.3 M Na-mannitol, 1 mM potassium-EDTA buffered to pH 7.4 with KOH). Further procedures took place at 4°C. The tissue (approximately 12 g) was homogenized (10 strokes, loose-fitting Dounce type homogenizer) in a total volume of 100 ml of mannitol-EDTA, then diluted to 300 ml. The homogenate was centrifuged (15 min, 2500 × g), the pellet discarded, and the supernatant further centrifuged (20 min, 20,000 × g). This pellet was resuspended in 30 ml of mannitol-EDTA, and 5-ml portions were layered over six discontinuous density gradients comprising 10-ml layers of 20, 16, 12, 8, and 2% Ficoll (w/v) in mannitol-EDTA. The gradients were spun in two SW25.2 rotors at 22,000 rpm for 100 min. Material equilibrated at the 8 to 12% interfaces was removed (approximately 60 ml), diluted with mannitol-EDTA to 200 ml, and centrifuged (20 min, 2500 × g). The sedimented material (synaptosomal membranes) was isolated by resuspension in 15 ml of 5 mM Tris-HCl, pH 7.4, 1 mM K-EDTA, followed by homogenization (Dounce, three strokes). After dilution to 10 ml in the same low osmolarity buffer, lysis was completed by stirring for 30 min. Finally the lysate was centrifuged (20 min, 25,000 × g) and the pellet resuspended in 5 ml of 0.32 M sucrose, 1 mM MgSO4, 0.5 mM K-EDTA, 5 mM Tris-SO4, pH 7.4. This material, containing 7 to 8 mg/ml of particulate protein, was stored in liquid nitrogen, and is referred to as membrane vesicles in this article.

For some experiments, membrane vesicles were further purified by density gradient centrifugation. Fresh membrane vesicles, prepared as above, except finally resuspended in 1 ml of sucrose-EDTA (0.32 M sucrose, 1 mM K-EDTA, pH 7.4), were layered over two discontinuous density gradients comprising 12-ml layers of 1.3, 1.1, 0.9, and 0.7 M sucrose in 1 mM K-EDTA, pH 7.4. The gradients were centrifuged in an SW25.2 rotor at 25,000 rpm for 45 min. Material sedimented to the 0.7 to 0.9 M and the 0.9 to 1.1 M sucrose interfaces was pooled (referred to as light membrane vesicles). Material at the 1.1 to 1.3 M sucrose interface was also collected (referred to as heavy membrane vesicles). Each fraction was diluted to a total of 200 ml with sucrose-EDTA and centrifuged (30 min, 25,000 × g). Light and heavy membrane vesicles were washed in 2.0 ml and 0.5 ml of sucrose-EDTA containing 5 mg/ml and 10 mg/ml of particulate protein, respectively, and stored frozen in liquid nitrogen. The light membrane vesicle fraction corresponds to the synaptic plasma membrane fraction isolated from synaptosomes by Crompton (25). By electron microscopy, this fraction appeared similar to the unfractionated membrane vesicles, containing a high proportion of vesicular membrane structures of varying size (0.1 to 0.35 μm) with few mitochondria or mitochondrial fragments. The heavy membrane vesicle fraction contained a higher proportion of mitochondria (either intact or predominantly swollen and partly devoid of internal structure) and a reduced content of single-membrane (nonmitochondrial) vesicles or fragments.

**Ca**²⁺ Influx and Efflux Experiments—All media present during pre-equilibration, Ca**²⁺** influx, and Ca**²⁺** efflux contained 1 mM MgSO4, 5 mM Hepes buffered to pH 7.4 with Tris (approximately 2.7 mM), and either 100 mM NaCl, KCl, LiCl, RbCl, NH4Cl, choline Cl, or 175 mM KCl (designated henceforth as Na**⁺** medium, K**⁺** medium, etc.) unless stated otherwise.

For Ca**²⁺** influx experiments, aliquots of membrane vesicles (100 μl) were thawed at room temperature, diluted with 1.0 ml of appropriate medium, and allowed to pre-equilibrate for 10 min at 37°C. Vesicles (10 μl) were then resuspended in 0.5 ml of 1.0 M sucrose ¹ of equilibration medium, and finally resuspended with 200 to 400 μl of the same medium. Ca**²⁺** influx was initiated by adding 5 μl of pre-equilibrated vesicles to 100 μl of appropriate external medium containing the additions as described in figures, together with 0.5 μCi of ⁴⁰CaCl₂ (approximately 500,000 cpm) and 10 μM unlabeled CaCl₂ (unless otherwise stated) to give a specific activity for Ca**²⁺** of 0.5 Ci/mmol. Influx continued at room temperature for the time specified and was terminated by dilution with 2.5 ml of ice-cold 0.15 M NaCl and rapid filtration through cellulose acetate membrane filters (0.5 μm pore size). After further washing with 2.5 ml of KCl, filters were dissolved in scintillation fluid and counted.

For Ca**²⁺** efflux experiments, vesicles were pre-equilibrated as above, except they were finally resuspended in 50 μl of the pre-equilibration medium. Influx proceeded in the same single vessel as pre-equilibration after addition of 1.0 ml of the appropriate external medium containing 10 μM CaCl₂ (with 0.5 Ci/mmol of ⁴⁰Ca) and continued for 20 min at room temperature. Ca**²⁺** efflux was initiated by diluting 20-μl aliquots of the ⁴⁰Ca-loaded vesicles into 500 μl of appropriate external efflux medium containing 10 μM CaCl₂ (with 0.5 Ci/mmol of ⁴⁰Ca) and continued at room temperature until termination and counting as for influx.

For both influx and efflux experiments, results are expressed as the amount of Ca**²⁺** accumulated by vesicles with nonspecific Ca**²⁺** adsorption to filters subtracted. The latter usually comprised 0.1% of the total Ca**²⁺** present.

**Measurement of Internal Volume**—The internal volume of membrane vesicles was measured by a modification of the procedure described by Padan et al. (26), using [methoxy-¹⁴C]inulin to determine the external volume, and H₂O to measure the total volume. Membrane vesicles (approximately 1 mg of particulate protein) were incubated in 1 ml of medium (22°C) until approximately 10 μCi of H₂O (1.8 Ci/mmol) and 50 μCi [methoxy-¹⁴C]inulin (40 mCi/mol) with approximately 10 μM unlabeled inulin to reduce binding of [methoxy-¹⁴C]inulin to membrane vesicles. After incubation, the membrane vesicles were centrifuged (20,000 × g, 2 min), the pellet dissolved in 1.0 ml of 19 Triton X-100, and counted along with samples of the original incubation mixture. From the corrected counts, internal volume was calculated as described (26), and found to be 1.1 μl/mg of particulate protein.

**Miscellaneous Procedures and Materials**—Protein measurements on membrane vesicle fractions were by the method of Lowry et al. (27) against standards of crystalline bovine serum albumin after 1-h treatment with 0.3% NaOH.

[⁴⁰CaCl₂], H₂O, and [methoxy-¹⁴C]inulin were obtained from New England Nuclear; [H]TPP (2.5 Ci/mmol), as the Br⁻ salt, was a generous gift from Dr. A. Liebmam, Hoffmann-La Roche, Nutley, N. J.; ATP (disodium salt), ruthenium red, oligomycin, rotenone, 2,4-dinitrophenol, ouabain, veratridine, mensyl acid, tetracaine HCl, AMP-PNP (tetralithium salt), and EDTA (dipotassium salt) were obtained from Sigma Chemical Co.; valinomycin was from ICN Pharmaceuticals; D600 HCl was from Knoll AG, Ludwigshafen, West Germany; sodium orthovanadate (Na₃VO₄·10H₂O) was from Fisher Scientific Co.; A23187 was a generous gift from Dr. R. A. Din, the Rockefeller College of Medicine and Veterinary Science, New York, N.Y.; batrachotoxin was a generous gift from Dr. J. W. Daly, National Institutes of Health, Bethesda, Md. All other chemicals were of reagent or higher grade. Filters used for the separation of vesicles were obtained from Millipore (type EHWP).

All additions to Ca**²⁺** influx and efflux experiments were in 0.5% of the final volume in either water or ethanol. ATP was neutralized with NaOH to pH 7.0 before addition.

**RESULTS**

Unlike intact synaptosomes, membrane vesicles are almost entirely devoid of internal organelles (24) which may themselves accumulate Ca**²⁺** and therefore render interpretation of uptake data difficult. Furthermore, since synaptosomal membrane vesicles are known to rapidly accumulate and release, for example, Na**⁺** ions (28), they may equilibrate with the desired internal medium, then dilute into appropriate external medium in which Ca**²⁺** influx proceeds.

By this procedure, membrane vesicles pre-equilibrated with Na**⁺** medium (see "Experimental Procedures") rapidly accumulated Ca**²⁺** except when the outside medium also contained Na**⁺** (Fig. 1). Thus only an outward concentration gradient of Na**⁺** (that is, net Na**⁺** efflux) caused Ca**²⁺** influx, most likely
reflecting a mediated Na⁺-Ca²⁺ exchange. The ionic composition of the external medium influenced both the rate and extent of Ca²⁺ uptake. In the presence of external K⁺, Rb⁺, or NH₄⁺ media, Ca²⁺ accumulation was very rapid, reaching half the equilibrium level between 1 and 2 min. External sucrose, choline, or Li⁺ media resulted in a slower rate of Ca²⁺ accumulation, and in the case of Li⁺, approximately half the maximal equilibrium Ca²⁺ uptake seen with K⁺. The Ca²⁺ ionophore A23187 prevented accumulation of Ca²⁺ by Na⁺ exchange, the low level of Ca²⁺ associated with vesicles in the presence of ionophore probably reflecting passive equilibration of Ca²⁺ with the internal space and/or binding of Ca²⁺ to the membrane surfaces. Using the estimate of internal volume (1.1 µl/mg of protein) as described under “Experimental Procedures,” the internal space within the 100-µl reaction volume was approximately 0.04 µl (in Fig. 1, each tube contained 35 µg of particulate protein). Of the total ⁴⁵Ca added, approximately 0.15% (excluding that bound to the filter) was associated with filtered vesicles in the presence of A23187. If this component represented internal free Ca²⁺ in passive equilibrium with the external medium, the internal space within each reaction tube would be 0.15 µl. This latter estimate of volume probably exceeds the former as a consequence of Ca²⁺ binding to the vesicles which may thus account for approximately 70% of the passive Ca²⁺ uptake. Therefore, the apparent 30-fold increase in internal Ca²⁺ concentration resulting from exchange with internally loaded Na⁺ may actually be closer to 100-fold above the external Ca²⁺ concentration.

Ca²⁺ uptake by membrane vesicles was stimulated by ATP (Fig. 2). In this experiment, the presence of 1 mM ATP gave rise to an internal Ca²⁺ concentration over 10-fold that in the absence of ATP. The rate of Ca²⁺ uptake appeared distinct from that occurring by Na⁺-Ca²⁺ exchange, final equilibrium not being attained before 30 min (as opposed to 5 min for Na⁺-Ca²⁺ exchange). However, as with Na⁺-Ca²⁺ exchange, the external medium influenced ATP-dependent Ca²⁺ uptake. Thus, the rate of ATP-dependent Ca²⁺ entry in the presence of external K⁺ medium was approximately 1.5-fold that in the presence of Na⁺ medium, this ratio remaining constant with time.

Influx of Ca²⁺ into the squid axon (1, 3, 4) or intact synaptosome (8, 9) may occur in response to depolarization of the resting potential across the plasma membrane. In order to determine the relationship between membrane potential and Ca²⁺ transport in synaptosomal membrane vesicles, the permeant, lipophilic cation [²³H]TPP⁺ was utilized. This ion is shown to enter membranes and equilibrate according to electrical potential so that its accumulation within cells indicates increased, interior-negative, transmembrane potential (29). Uptake of [²³H]TPP⁺ was measured simultaneously with Ca²⁺ accumulation using membrane vesicles pre-equilibrated with K⁺ medium, under various external ionic conditions. With external Na⁺ there was a slight increase in [²³H]TPP⁺ uptake (and hence membrane potential) relative to that in the continued presence of K⁺, presumably due to the lower permeability of Na⁺ relative to K⁺ (Fig. 3B). External Na⁺ was also associated with decreased Ca²⁺ uptake in this experiment (Fig. 3A). However, the large increase in membrane potential occurring in the presence of external sucrose (uncharged and relatively nonpermeant), rather than further inhibiting Ca²⁺ movement, instead resulted in slightly higher Ca²⁺ uptake relative to external K⁺ (Fig. 3, A and B). Similarly, the K⁺-specific ionophore, valinomycin, which by increasing the selective permeability of the membranes to K⁺ transiently gave a large increase in K⁺ influx (in the absence of external K⁺) and hence in membrane potential, did not inhibit Ca²⁺ influx (Fig. 3, A and B). Rather, there was a small although consistent increase in Ca²⁺ influx associated with valinomycin-in-
Membrane vesicles were all pre-equilibrated in K⁺ medium for 10 min at 37°C. Uptake was initiated by addition of 5 µl of pre-equilibrated membrane vesicles to 100 µl of external medium which was either Na⁺ medium (C), K⁺ medium (D), or sucrose medium (E), each containing 10 µM CaCl₂, 0.5 Ci/mmol of ⁴⁵Ca, and 5 µM [⁸⁶H]TPP⁺ (2.5 Ci/mmol). Details of the incubation conditions and media are described under "Experimental Procedures." In some cases 50 µM valinomycin was added (as 0.5 µl of ethanol) after 3 min of incubation (D, E). Uptake values shown for Ca²⁺ (A) and TPP⁺ (B) were calculated from counts recovered by filtration, with nonspecific binding to the filters subtracted, and with spillover of ⁴⁵Ca into ³H (9%) corrected for.

An important question concerning the two major mediated Ca²⁺ transport mechanisms (Na⁺-Ca²⁺ exchange and ATP-dependent Ca²⁺ uptake) is whether both co-exist in the same vesicles or whether distinct populations of vesicles containing only one transport mechanism derived from separate organelles, exist. These two possibilities were resolvable since Na⁺-Ca²⁺ exchange resulting from Na⁺ influx rather than any change in membrane potential. Since A23187 reduced Ca²⁺ accumulation to the same level (approximately 0.15 mmol of Ca²⁺/mg of protein) in the presence of external K⁺, Na⁺, or sucrose (data not shown), changes in either Ca²⁺ binding or vesicle volume were not a significant cause of changes in overall Ca²⁺ accumulation.

Arising from these observations is the question of origin of such vesicles. The possibility that membrane vesicles may reflect mitochondrial Ca²⁺ uptake (known to be influenced by ATP and Na⁺) was investigated by comparing Ca²⁺ uptake in membrane vesicles with that in the light and heavy vesicle fractions isolated by density gradient centrifugation as described under "Experimental Procedures." Heavy membrane vesicles (observed by electron microscopy to contain a high proportion of mitochondria) gives 30% of the ATP-dependent Ca²⁺ uptake observed with an equal quantity of the light vesicle fraction (Fig. 5). Furthermore, ruthenium red (10 µM), a potent inhibitor of mitochondrial Ca²⁺ transport (14, 15), reduced uptake into unfractionated, light, and heavy vesicles by only 20, 25, and 45%, respectively (Fig. 5, A, C, and E). These results suggest; firstly, unfractionated vesicles reflect only a small proportion of mitochondrial Ca²⁺ uptake; secondly, the heavy vesicle fraction may be more contaminated with mitochondria, although it probably also contains increased amounts of non-Ca²⁺-accumulating particles; thirdly, fractionation is not advantageous in selectively purifying Ca²⁺ uptake activity probably due to denaturation of vesicles as a result of lengthy purification. Na⁺-Ca²⁺ exchange activity in the three fractions (Fig. 5, B, D, and F) very closely correlated with the ruthenium red-insensitive ATP-dependent Ca²⁺ uptake activity, supporting the above conclusions, and reinforcing the conclusion that a single population of vesicles contains both Ca²⁺ transport mechanisms.

High concentrations of other mitochondrial inhibitors influenced ATP-dependent Ca²⁺ uptake by membrane vesicles as expected relative to ruthenium red (Table I). Thus rotenone and cyanide were with little effect, ATP increasing mitochondrial Ca²⁺ uptake independently of any substrate oxidation. 2,4-Dinitrophenol and azide (uncoupling and therefore opposing the utilization of ATP for ion translocation) reduced Ca²⁺ uptake by approximately 15%. Oligomycin, effective in directly inhibiting mitochondrial ATP utilization, gave similar inhibition (approximately 20%) as ruthenium red. None of these agents were with significant effect upon Na⁺-Ca²⁺ exchange (Table I).

Na⁺-Ca²⁺ exchange was selectively inhibited by agents able...
However, batrachotoxin, a similarly active agent, was without effect. ouabain. 

\[ 
\text{Na}^+ - \text{Ca}^{2+} \text{ exchange was independent of the Na}^+ \text{ channel.} 
\]

Details of the media and incubation conditions are given under "Experimental Procedures." The concentrations of membrane protein during Ca\(^{2+}\) uptake were 0.10, 0.07, and 0.09 mg/ml for unfractionated, light, and heavy membrane vesicles, respectively.

to dissipate Na\(^+\) gradients (Table I). For example, the Na\(^+\) ionophore, monensin, inhibited Ca\(^{2+}\) uptake, as did (although less effectively) the Na\(^+\) channel opening agent, veratridine. However, batrachotoxin, a similarly active agent, was without effect. Na\(^+\)-Ca\(^{2+}\) exchange was independent of the Na\(^+\) channel blocker, tetrodotoxin, and the (Na\(^+\) + K\(^+\))-ATPase inhibitor, ouabain.

Several agents reported to be inhibitors of ATP-dependent Ca\(^{2+}\) uptake gave large effects, although these were not necessarily specific to this activity (Table I). Orthovanadate (30) and mersalyl (15, 31) were potent inhibitors of ATP-dependent Ca\(^{2+}\) uptake, although the latter also considerably reduced Na\(^+\)-Ca\(^{2+}\) exchange. Tetracaine also inhibited both processes probably as a result of lipid modulation (32) rather than possible direct inhibition of the Ca\(^{2+}\) transport system (15, 21). The nonhydrolyzable ATP analogue, AMP-PNP, inhibited ATP-dependent Ca\(^{2+}\) uptake most likely by irreversible association with the ATPase enzyme, although its partial effect upon Na\(^+\)-Ca\(^{2+}\) exchange is unclear. Significant is the absence of effect of the Ca\(^{2+}\) channel antagonist, D600, upon either Ca\(^{2+}\) uptake process.

The dependence of Ca\(^{2+}\) uptake upon ATP concentration was investigated using different external and internal media (Fig. 6). Under all conditions, ATP appeared to have a biphasic effect, low concentrations enhancing Ca\(^{2+}\) uptake whereas concentrations above 0.3 or 1.0 mM proving inhibitory. When the external medium contained either K\(^+\) or sucrose, the \(K_a\) for ATP with respect to the stimulatory phase of Ca\(^{2+}\) uptake was approximately 40 \(\mu M\) (Fig. 6, B and O). This value increased to approximately 500 \(\mu M\) when the external medium contained Na\(^+\), regardless of the pre-equilibration medium. External K\(^+\) increased the \(V_{\text{max}}\) for Ca\(^{2+}\) uptake by approximately 50% relative to external Na\(^+\) or sucrose. An inwardly directed Na\(^+\) gradient, as expected, reduced the \(V_{\text{max}}\) for Ca\(^{2+}\) uptake, since accumulated Ca\(^{2+}\) would initially be subject to Na\(^+\)-dependent efflux. An outwardly directed Na\(^+\) gradient resulted in a constant increment in Ca\(^{2+}\) uptake (as seen by the difference between uptake curves for Na\(^+\)/K\(^+\) and K\(^+\)/K\(^+\) in Fig. 6A) such that the ATP-dependent component of Ca\(^{2+}\) uptake (plotted in Fig. 6B) was independent of the component due to exchange with Na\(^+\), up to ATP concentrations giving maximal Ca\(^{2+}\) accumulation (0.3 mM). This additivity of action suggests that the two mechanisms of Ca\(^{2+}\) transport are distinct and function independently even though they coexist within a single membrane. The inhibitory phase of ATP was partially attributable to Na\(^+\) added with ATP (approximately 3 Na\(^+\)/ATP), although may also result from chelation of Ca\(^{2+}\) by ATP. The latter mechanism may account for the inhibition of Na\(^+\)-Ca\(^{2+}\) exchange by AMP-PNP (Table I) where use of the Li\(^+\) salt of the nucleoside triphosphate precludes the possibility of a Na\(^+\) effect.

Although Na\(^+\)-Ca\(^{2+}\) exchange and ATP-dependent Ca\(^{2+}\) uptake were revealed as separate mechanisms, both showed similar temperature dependence (Fig. 7). Thus the initial rate

### Table I

**Influence of mitochondrial inhibitors or agents modifying Na\(^+\) or Ca\(^{2+}\) flux on Na\(^+\)-Ca\(^{2+}\) exchange and ATP-dependent Ca\(^{2+}\) uptake by synaptosomal membrane vesicles**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Na-Ca exchange</th>
<th>ATP-dependent Ca uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>2.43 ± 0.15</td>
<td>100</td>
</tr>
<tr>
<td>Ethanol (0.5%)</td>
<td>2.24 ± 0.11</td>
<td>92</td>
</tr>
<tr>
<td>A23187 (10 (\mu M))</td>
<td>0.17 ± 0.06</td>
<td>9</td>
</tr>
<tr>
<td><strong>Mitochondrial inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotenone (12 (\mu M))</td>
<td>2.43 ± 0.04</td>
<td>100</td>
</tr>
<tr>
<td>KCN (0.1 mM)</td>
<td>2.48 ± 0.15</td>
<td>102</td>
</tr>
<tr>
<td>2,4-Dinitrophenol (0.1 mM)</td>
<td>2.54 ± 0.35</td>
<td>105</td>
</tr>
<tr>
<td>NaN(_3) (0.1 mM)</td>
<td>2.42 ± 0.26</td>
<td>100</td>
</tr>
<tr>
<td>Oligomycin (50 (\mu M))</td>
<td>2.23 ± 0.09</td>
<td>92</td>
</tr>
<tr>
<td>Ruthenium red (10 (\mu M))</td>
<td>2.37 ± 0.14</td>
<td>98</td>
</tr>
<tr>
<td>Na(^+) flux modifiers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monensin (8 (\mu M))</td>
<td>0.32 ± 0.02</td>
<td>13</td>
</tr>
<tr>
<td>Veratridine (185 (\mu M))</td>
<td>1.68 ± 0.06</td>
<td>69</td>
</tr>
<tr>
<td>Batrachotoxin (1 (\mu M))</td>
<td>2.37 ± 0.10</td>
<td>98</td>
</tr>
<tr>
<td>Tetrodotoxin (15 (\mu M))</td>
<td>2.42 ± 0.06</td>
<td>100</td>
</tr>
<tr>
<td>Ouabain (0.2 mM)</td>
<td>2.41 ± 0.07</td>
<td>99</td>
</tr>
<tr>
<td>Ca(^{2+}) flux modifiers</td>
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<td></td>
</tr>
<tr>
<td>Orthovanadate (1 (\mu M))</td>
<td>1.97 ± 0.03</td>
<td>81</td>
</tr>
<tr>
<td>Mersalyl (1 mM)</td>
<td>0.93 ± 0.06</td>
<td>38</td>
</tr>
<tr>
<td>Tetracaine HCl (3 (\mu M))</td>
<td>0.95 ± 0.03</td>
<td>59</td>
</tr>
<tr>
<td>AMP-PNP (2 mM)</td>
<td>1.47 ± 0.19</td>
<td>60</td>
</tr>
<tr>
<td>D600 (0.2 mM)</td>
<td>2.54 ± 0.13</td>
<td>105</td>
</tr>
</tbody>
</table>

*Added in 0.5 \(\mu l\) of ethanol.*
Calcium Transport in Synaptosomal Membrane Vesicles

ATP

FIG. 6. ATP-dependence of Ca\(^{2+}\) uptake into synaptosomal membrane vesicles. A, vesicles were pre-equilibrated in either Na\(^+\) medium (A, O), K\(^+\) medium (A), or sucrose medium (B). Ca\(^{2+}\) uptake took place with either external Na\(^+\) medium (A, O, Δ), K\(^+\) medium (O), or sucrose medium (O) containing 10 \(\mu\)M CaCl\(_2\) with 0.5 Ci/mmol of \(^{45}\)Ca, and the ATP concentration indicated. Incubation proceeded for 10 min at room temperature in each case. Details of media and incubation conditions were as described under "Experimental Procedures." B, ATP-dependent component of Ca\(^{2+}\) uptake data shown in A, that is, with Ca\(^{2+}\) uptake observed in the absence of ATP subtracted. C, Lineweaver-Burk plot of ATP-dependent Ca\(^{2+}\) uptake data shown in B.

FIG. 7. Influence of temperature upon Na\(^+\)-Ca\(^{2+}\) exchange and ATP-dependent Ca\(^{2+}\) uptake by synaptosomal membrane vesicles. A, membrane vesicles were pre-equilibrated in Na\(^+\) medium and Ca\(^{2+}\) uptake took place with external K\(^+\) medium containing 10 \(\mu\)M CaCl\(_2\) (with 0.5 Ci/mmol of \(^{45}\)Ca) at either 4°C (C), 22°C (O), or 37°C (Δ). B, membrane vesicles were pre-equilibrated in K\(^+\) medium and Ca\(^{2+}\) uptake took place as in A except the external medium also contained 1 mM ATP. Details of the composition of media and conditions for incubation are given under "Experimental Procedures." of Ca\(^{2+}\) uptake was in both cases reduced more than 10-fold by lowering the temperature from 22°C (standard condition) to 4°C. Although a moderate increase in both rates occurred upon elevation of incubation temperature to 37°C, significant efflux of accumulated Ca\(^{2+}\) occurred upon prolonged incubation at this temperature. The more rapid efflux of Ca\(^{2+}\) accumulated by Na\(^+\)-Ca\(^{2+}\) exchange relative to ATP-dependent Ca\(^{2+}\) uptake is probably due to the rapid elimination of the driving force (Na\(^+\) gradient) in the former case whereas the driving force of the latter (ATP) probably remains, albeit at reduced concentrations, for a longer period.

The specificities of the two Ca\(^{2+}\) uptake processes to other divalent cations and La\(^{3+}\) were quite distinct. For Na\(^+\)-Ca\(^{2+}\) exchange, Ca\(^{2+}\) and Sr\(^{2+}\) were equipotent in saturating the uptake process with \(K_{\text{Na}}\) values of approximately 40 \(\mu\)M (Fig. 8A), Ba\(^{2+}\) and Mn\(^{2+}\) were 10-fold and 25-fold less effective, respectively, although their inhibition curves were similar in shape. Mg\(^{2+}\) only inhibited Ca\(^{2+}\) uptake at concentrations greater than 1 mM. In the presence of 1 mM Mg\(^{2+}\) (standard condition), the specificity toward other divalent cations was identical (data not shown). For ATP-dependent Ca\(^{2+}\) uptake (measured in the presence of 1 mM Mg\(^{2+}\)), Ca\(^{2+}\) appeared to half-maximally saturate the process at approximately 12 \(\mu\)M (Fig. 8B). Sr\(^{2+}\), Mn\(^{2+}\), and Ba\(^{2+}\) were, respectively, 10-, 50-, and 1000-fold less effective.

FIG. 8. Cationic specificity of Na\(^+\)-Ca\(^{2+}\) exchange and ATP-dependent Ca\(^{2+}\) uptake by synaptosomal membrane vesicles. A, membrane vesicles were pre-equilibrated with Na\(^+\) medium, and Ca\(^{2+}\) uptake proceeded in external K\(^+\) medium containing 10 \(\mu\)M CaCl\(_2\) (with 0.5 Ci/mmol of \(^{45}\)Ca) at either 4°C (C), 22°C (O), or 37°C (Δ). B, membrane vesicles were pre-equilibrated with K\(^+\) medium, and Ca\(^{2+}\) uptake proceeded in external K\(^+\) medium containing 10 \(\mu\)M CaCl\(_2\) (with 0.5 Ci/mmol of \(^{45}\)Ca) with 1 mM ATP. Both media contained 1 mM MgSO\(_4\). C, Ca\(^{2+}\) uptake was exactly as in B except media were Mg\(^{2+}\)-free. D, uptake of Ca\(^{2+}\) was either by Na\(^+\)-Ca\(^{2+}\) exchange (C) exactly as in A, except in the presence of 1 mM MgSO\(_4\), or by ATP-dependent Ca\(^{2+}\) uptake (C) exactly as in B. Composition of media and incubation conditions were as described under "Experimental Procedures." In all experiments, influx of Ca\(^{2+}\) was for 15 min at room temperature in the presence of the appropriate concentration of di- or trivalent cation chloride as shown.
and 100-fold less effective at inhibiting uptake. In the absence of Mg²⁺, ATP-dependent Ca²⁺ uptake was reduced by 85% (Fig. 8C). The residual activity (which contained approximately one-half ATP-independent Ca²⁺ uptake) was saturated by Ca²⁺ half-maximally at approximately 100 μM. ATP-dependent Ca²⁺ uptake was dependent upon the presence of Mg²⁺ or Mn²⁺, each half-maximally stimulating uptake at approximately 100 μM. Mn²⁺ was inhibitory above 0.5 mM whereas Mg²⁺ only inhibited above 1 mM, the standard concentration for uptake. The inhibitory activity of La³⁺, known to replace Ca²⁺ in many binding and transport systems, was approximately 100-fold less sensitive to La³⁺.

**DISCUSSION**

The experiments demonstrate that synaptosomal membrane vesicles accumulate Ca²⁺ by two major mechanisms, Na⁺-Ca²⁺ exchange (Fig. 1) and ATP-dependent Ca²⁺ uptake (Fig. 2). In both cases, A23187 rapidly reverses uptake (Fig. 4) indicating an accumulation of free internal Ca²⁺ as opposed to Ca²⁺ bound to membrane surfaces. A third, minor component of Ca²⁺ uptake observed under conditions of increased [³H]TPP⁺ accumulation and probably reflecting hyperpolarization of the membrane potential (Fig. 3).

Na⁺-Ca²⁺ exchange is a fully reversible process, accumulated Ca²⁺ being effluxed upon treatment with external Na⁺ (Fig. 4A). Since Ca²⁺ accumulated by ATP-dependent Ca²⁺ uptake is also rapidly effluxed by external Na⁺ (Fig. 4B), it is concluded that a single population of vesicles exists containing both ATP-dependent Ca²⁺ transport and Na⁺-Ca²⁺ exchange mechanisms. The small component (approximately 20%) of Ca²⁺ not effluxed by Na⁺ (Fig. 4B) may indicate a subpopulation of vesicles containing only ATP-dependent Ca²⁺ transport. These conclusions are supported by the close correlation between ruthenium red-sensitive ATP-dependent Ca²⁺ uptake and Na⁺-Ca²⁺ exchange observed in the three fractions of membrane vesicles used in Fig. 5.

There is considerable evidence that the squid axon plasma membrane contains Na⁺-Ca²⁺ exchange, where its activity has been implicated in the removal of Ca²⁺ from the axoplasm (1). There is also good evidence that the plasma membrane of intact mammalian synaptosomes contains a similar Na⁺-Ca²⁺ exchange mechanism (1, 10–12). Therefore, the present data strongly suggest that the plasma membrane is the source of vesicles containing both Na⁺-Ca²⁺ exchange and ATP-dependent Ca²⁺ transport.

Although ATP-dependent Ca²⁺ uptake has been well established to operate in mitochondria (1, 3, 4, 14, 15), previous reports have been unable to locate the exact origin of nonmitochondrial ATP-dependent Ca²⁺ transport observed using synaptosomal lysates (13–15), brain microsomes (16–18), or intraterminal neurotransmitter secretory vesicles (19, 20). In the present experiments, using high concentrations of mitochondrial inhibitors, it is clear that the large majority (approximately 90%) of the ATP-dependent Ca²⁺ uptake is not due to mitochondria (Fig. 5, Table 1), this being supported by fractionation experiments (Fig. 5). It is unlikely that small secretory-like vesicles released from the nerve terminal account for ATP-dependent Ca²⁺ uptake since they are not expected to sediment at 20,000 × g. Furthermore, recent evidence suggests that secretory vesicles per se may not accumulate Ca²⁺, such activity arising from contamination with microsomes derived from other organelles (33). The problem of heterogeneity within microsomal fractions and synaptosomal lysates has been the obstacle in attempting to determine the site of intrasynaptosomal ATP-dependent Ca²⁺ uptake. However, Blaustein and co-workers have proposed that the synaptosomal endoplasmic reticulum is the site of such internal Ca²⁺ uptake (15–15). This view was formulated mainly from indirect evidence and by analogy with the Ca²⁺-sequestering properties of endoplasmic reticulum in some other cells, and in particular, the activity of sarcoplasmic reticulum in muscle. Recently, these workers have presented ultrastructural evidence to support the existence of an intracellular Ca²⁺-sequestering organelle (34). In contrast, the present report provides direct evidence for the location of synaptosomal ATP-dependent Ca²⁺ transport in the plasma membrane. However, the results do not preclude a small fraction of vesicles containing only ATP-dependent Ca²⁺ uptake originating from a separate organelle, perhaps the smooth endoplasmic reticulum.

It is well established that Ca²⁺ uptake into purified, intact synaptosomes is little affected by externally applied ATP (10, 13–15). Therefore it is concluded that a cytosolic-facing ATP-dependent Ca²⁺ transport site exists on the inside of the plasma membrane, and is exposed upon lysis by inversion of plasma membrane fragments to form Ca²⁺-accumulating vesicles analogous in function with inverted vesicles containing the Ca²⁺-Mg²⁺-ATPase from the erythrocyte plasma membrane (35). There is little doubt that at least a proportion of the membrane vesicles used in the present experiments are derived from the plasma membrane (24, 28), and there is evidence (Fig. 5) that the two Ca²⁺ transport mechanisms are specifically associated with a fraction enriched in plasma membranes (25). Furthermore, several factors suggest the formation of function of inverted plasma membrane vesicles. Firstly, the use of Ca²⁺- and Mg²⁺-free conditions (with 1 mM EDTA) during isolation, lysis, and rescaling of synaptosomes (see "Experimental Procedures") is a condition known to selectively favor the formation of inverted plasma membrane vesicles from erythrocytes (36). Secondly, hyperpolarization of the membrane vesicles (Fig. 3) is associated with a slight increase in Ca²⁺ accumulation, whereas increased influx of Ca²⁺ into the intact synaptosome is associated with depolarization of the membrane potential (9). If a voltage-sensitive Ca²⁺ channel operates in the synaptic plasma membrane allowing Ca²⁺ entry in response to a depolarizing impulse (9), then the data suggest that the vesicles may contain an abundance of inverted structures although it should be noted that Ca²⁺ might be expected to move into the vesicles as a direct result of the membrane potential created in this experiment. Thirdly, the Ca²⁺ channel antagonist, D600, was without effect on any of the Ca²⁺ transport processes (Table 1) consistent with evidence that this agent may act selectively on the outside of the plasma membrane (37). Lastly, tetanus toxin, which has been shown to bind to the outer surface of synaptosomes (29, 38) and to enhance Ca²⁺ efflux, is without effect on Ca²⁺ movement using membrane vesicles. However, sonication of vesicles in the presence of tetanus toxin results in increased Ca²⁺ uptake (selectively via Na⁺-Ca²⁺ exchange), suggesting that this treatment allows entry of the toxin presumably to an internal site that originally existed on the outer surface of the intact synaptosome.

The use of membrane vesicles prepared as described appears fortuitous in revealing a high content of nonmitochondrial ATP-dependent Ca²⁺ uptake. Using a fresh synaptosomal lysate prepared in the presence of 1 mM MgCl₂, Blaustein and co-workers observed that 95% of the ATP-dependent Ca²⁺

uptake was into mitochondria (inhibited by, for example, 10 mM ruthenium red) (13-15). It has been suggested above that the absence of divalent cations during preparation of the present vesicles increases exposure of the ATP-dependent Ca\(^{2+}\) transport site. In addition, our use of prefrozen vesicles appears to selectively inhibit mitochondrial ATP-dependent Ca\(^{2+}\) uptake.\(^3\)

Recent evidence supports the conclusion that both Na\(^+-\)Ca\(^{2+}\) exchange and ATP-dependent Ca\(^{2+}\) transport mechanisms exist within the plasma membrane of excitable cells. Firstly, although (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity is well documented in various subcellular fractions from the brain (18, 19, 21-23), recent evidence suggests that the isolated synaptic plasma membrane contains this activity (39). Secondly, an ATP-dependent Ca\(^{2+}\) extrusion mechanism in the squid axon plasma membrane has recently been characterized by DiPolo (5) and DiPolo and Beaugé (6), this membrane therefore containing the same two Ca\(^{2+}\) transport mechanisms suggested in the present report to coexist in the synaptosomal plasma membrane. Interestingly, ATP-depends Ca\(^{2+}\) transport in the squid axon is selectively inhibited by orthovanadate (30), the activity of which in the present report (Table I) suggests the same ATP-dependent mechanism transports Ca\(^{2+}\) in the mammalian nerve terminal. Lastly, the sarcolemma of muscle has been shown to contain a very similar Na\(^+-\)Ca\(^{2+}\) exchange mechanism (40, 41), and more importantly, experimental evidence similar to the present report has very recently suggested that the sarcolemma also contains an ATP-dependent Ca\(^{2+}\) transport mechanism (42).

The present report provides some information regarding the mechanism and regulation of the co-residing Ca\(^{2+}\) transport activities. With regard to Na\(^+-\)Ca\(^{2+}\) exchange, the full reversibility (Fig. 4A) suggests a non sided antipporter mechanism mediating the transfer of Na\(^+\) or Ca\(^{2+}\). It is distinct from the Na\(^+\) channel since tetrodotoxin is noninhibitory (Table 1), but is specifically inhibited by those agents dissipating a Na\(^+\) gradient (monensin, veratridine). The enhancement by peritoned monovalent cations (other than Na\(^+\) and Li\(^+\), Fig. 1) may indicate the involvement of charge transfer in Na\(^+-\)Ca\(^{2+}\) exchange. Li\(^+\) may inhibit the process by partially replacing Na\(^+\), otherwise high specificity exists for both monovalent (Fig. 1) and divalent cation involvement (Fig. 8A).

The ATP-dependent Ca\(^{2+}\) transport activity most likely follows the action of a cytosolic-facing (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase since uptake is dependent upon the presence of both ATP and Mg\(^{2+}\) (or Mn\(^{2+}\), Fig. 8C) and is inhibited by the nonhydrolizable analogue AMP-PNP (Table I). Vanadate is a relatively specific inhibitor whereas other inhibitors tested are nonspecific, affecting Na\(^+-\)Ca\(^{2+}\) exchange as well. Like Na\(^+-\)Ca\(^{2+}\) exchange, the mechanism is dependent on temperature (Fig. 7) and influenced by the external ionic composition, being enhanced with external K\(^+\) (Figs. 2 and 6A).

The two Ca\(^{2+}\) transport activities are independent mechanisms. This is concluded from their additivity of action (Fig. 6, A and B), their different divalent cation specificities (Fig. 8, A and B), their remarkably distinct sensitivities to La\(^{3+}\) (Fig. 8D), and the differential effects of mediators of Na\(^+\) flux and orthovanadate (Table I). Although independent, Na\(^+\) (without any net Na\(^+\) flux) inhibits the \(K_a\) for ATP of ATP-dependent Ca\(^{2+}\) uptake by almost 10-fold (Fig. 6, B and C). It is possible that changes in intracellular Na\(^+\) or ATP resulting, for example, from depolarization and/or utilization of ATP within the cytosol, may thus be controlling influences on Ca\(^{2+}\) efflux from the nerve terminal via the two Ca\(^{2+}\) transport mechanisms.

\(^3\)D. L. Gill, E. F. Grollman, and L. D. Kohn, unpublished observations.

The apparent affinities of Na\(^+-\)Ca\(^{2+}\) exchange and ATP-dependent Ca\(^{2+}\) uptake are approximately 40 and 12 \(\mu\)M, respectively (Fig. 8, A and B). Recently, using Ca\(^{2+}\) concentrations approximately buffered with EGTA, the affinity of nonmitochondrial ATP-dependent Ca\(^{2+}\) uptake in synaptosomal lysates has been measured at approximately 0.5 \(\mu\)M (43). The discrepancy arises from the use of EGTA buffers, almost identical differences being observed for the activation of (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase by Ca\(^{2+}\) in ressealed erythrocyte ghosts (44) and for the uptake of Ca\(^{2+}\) by inverted erythrocyte ghosts (45), in both cases using either directly added or buffered Ca\(^{2+}\) concentrations. The difficulty may be explained by the uncertainty of stability constant values for the EGTA-Ca\(^{2+}\) complexes under experimental conditions (46) and/or the possible recognition of chelated Ca\(^{2+}\) by the Ca\(^{2+}\) transporting site (45). Using EGTA-Ca\(^{2+}\) buffers, precisely calculated and prepared, we have observed similar discrepancies, and submit to the view that affinity measured by direct Ca\(^{2+}\) addition may be the more realistic.

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