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Orientation of synaptic plasma membrane vesicles containing calcium pump and sodium-calcium exchange activities

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Sidedness of synaptic plasma membrane vesicles isolated from brain synaptosomes has been assessed by two distinct experimental approaches: first, analysis of $(Na^+ + K^+)$-ATPase, Mg^{2+}-ATPase, and $(Ca^{2+} + Mg^{2+})$-ATPase activities before and after permeabilization of vesicles; second, analysis of Ca^{2+} fluxes via the Na^+/Ca^{2+} exchanger, before and after modification of an imposed Na^+ gradient by penetrating or nonpenetrating Na^+ channel-modifying drugs. 0.05% saponin, which completely permeabilizes the vesicles, increases digitoxigenin-sensitive $(Na^+ + K^+)$-ATPase, basal Mg^{2+}-ATPase, and $(Ca^{2+} + Mg^{2+})$-ATPase activities by 51.0, 47.4, and 83.6%, respectively. Saponin increases only the V_{max} of the latter activity, the K_m for Ca^{2+} (0.13 μM; the same as that for Ca^{2+}-pumping) being unaltered by saponin. An increment of 20.5% in the V_{max} of $(Ca^{2+} + Mg^{2+})$-ATPase activity with 10 μM A23187, reveals that the enzyme activity in nonpermeabilized vesicles is limited by the formation of a Ca^{2+} gradient. Thus, the saponin-induced increment in $(Ca^{2+} + Mg^{2+})$-ATPase due only to exposure of occluded sites (as opposed to Ca^{2+} gradient dissipation) is actually 52%, which is similar to values for both other ATPases, and suggests that 32–35% of plasma membranes exist in an inverted orientation. Vesicle orientation was independently assessed by the differential actions of tetrodotoxin (a membrane impermeant blocker) and veratridine (a membrane permeant agonist) on Na^+-channel opening measured indirectly by dissipation of an imposed Na^+ gradient utilized to drive a large $^{45}Ca^{2+}$ accumulation via the Na^+/Ca^{2+} exchanger. Tetrodotoxin reverses 35–44% of veratridine-mediated Na^+ gradient-dissipation, the relative membrane-permeability of the two channel modifiers, suggesting that 56–65% of sealed vesicles are inverted. The concurrence of these two independent measurements of vesicle orientation reinforces their validity.

Introduction

The plasma membrane of resting neural cells separates and maintains a 10000-fold concentration gradient of calcium ions which is of fundamental importance to Ca^{2+} signaling within such cells [1–3]. The efflux of Ca^{2+} across the neural plasma membrane has been studied extensively [3–10]. Two major Ca^{2+} transport mechanisms have been identified in this membrane using a preparation of synaptic plasma membrane vesicles isolated from purified brain synaptosomes [6]. Thus both an $(ATP + Mg^{2+})$-dependent Ca^{2+} pump and a Na^+/Ca^{2+} exchanger have been shown to coexist within a single population of vesicles, each mechanism being capable of accumulating a high con-

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Elon, *p*-(methylamino)phenol sulfate.

centration of intravesicular Ca^{2+} [3,6]. The plasma membrane origin of the two mechanisms has been concluded from further studies which revealed that the same vesicles also contain both Na^+ channel and ATP-dependent Na^+-pumping activities [9]. Recent studies have revealed that both of the synaptic plasma membrane Ca^{2+} transport mechanisms have high affinity for Ca^{2+}, with K_m values for Ca^{2+} of 0.1 and 0.5 μM for the ATP-dependent Ca^{2+} pump and Na^+/Ca^{2+} exchanger, respectively [10]. These studies have further revealed that the two mechanisms differ with respect to their Ca^{2+} saturation kinetics, the Ca^{2+} pump displaying simple saturation of a single class of Ca^{2+} transport sites whereas the Na^+/Ca^{2+} exchanger revealing complex kinetics with saturation over a wide range of free Ca^{2+} concentrations indicating the existence of an additional low-affinity Ca^{2+} transporting component of the exchanger saturating at millimolar Ca^{2+} concentrations [10].

Since our studies have revealed that Na^+-Ca^{2+} exchange across the synaptic plasma membrane is a fully reversible process [6], one attractive explanation for the existence of more than one Ca^{2+} transporting site for this mechanism is the exposure of discrete sites derived from the two plasma membrane surfaces [10]. Such a hypothesis would suppose a heterogeneous orientation of the synaptic plasma membrane vesicles with a significant proportion of both inverted and noninverted structures. It was evident from experiments on both Ca^{2+}-pumping [6] and Na^+-pumping activities [9], that a significant fraction of inverted plasma membrane vesicles exist within the preparation. However, neither the existence of noninverted vesicles nor the relative abundance of vesicles of either oritentation had been assessed.

The present report describes some novel experiments which utilize functional characteristics of each of the two major Ca^{2+} translocating mechanisms (the Ca^{2+} pump and Na^+/Ca^{2+} exchanger) and the two major Na^+-translocating mechanisms (the Na^+ pump and Na^+ channel), in independent studies to assess the relative proportions of plasma membrane vesicles of either orientation. The experiments provide concordant evidence for the existence of both inverted and noninverted sealed plasma membrane vesicles within the preparation in the approximate proportions of 2 to 1, respectively.

Materials and Methods

Preparation of synaptic plasma membrane vesicles. Plasma membrane vesicles were prepared from purified synaptosomes isolated from cerebral cortex of 2-month, male CD rats (Charles River) as previously described [6,10]. The procedure involves the purification of isolated synaptosomes using methodology based on that of Cotman [11] as modified and described by Kanner [12]. Vesicle formation by hypotonic lysis and subsequent purification of vesicles were as described before [6]. The vesicles were finally resuspended in 0.32 M sucrose/1 mM EDTA-KOH (pH 7.4) and rapidly frozen in liquid nitrogen and stored at −95°C.

Measurement of ATPase activities. The enzymatic hydrolysis of ATP was measured by the spectrophotometric determination of released inorganic phosphate using an assay similar to that described by LeBel et al. [13]. Basal Mg^{2+}-dependent ATPase activity was measured in the presence of 150 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) containing 0.2 to 0.8 mM EGTA-Tris, 2.5 mM ATP-Tris, and 100 μM dithiothreitol. Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activity was measured under identical conditions except in the presence of the indicated concentration of free Ca^{2+}, precisely buffered with EGTA as described previously [10]. $(Na^+ + K^+)$-dependent ATPase activity was measured in the presence of 135 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) containing 2.5 mM ATP-Tris, and 100 μM dithiothreitol, either with or without 250 μM digitoxigenin. This Na^+-pump inhibitor was chosen since its hydrophobicity permits penetration of the molecule within closed plasma membrane vesicles [14]. Under all conditions, ATPase incubations were initiated by addition of 10 μl of synaptic plasma membrane vesicles (washed and resuspended in 150 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4)) containing 0.030–0.035 mg protein, to give a total assay volume of 400 μl. Incubation was continued for 5 or 10 min at 37°C, and terminated by addition of 200 μl of 10% trichloroacetic acid at 0°C. To each tube was added 1.5 ml of 10 mM $CuSO_4$, 34 mM sodium acetate, 2 M acetic acid (pH 4.0), 0.25 ml of 0.4% ammonium molybdate, and 0.25 ml of 2%

Elon, followed by thorough vortexing. After 15 min at 22°C to permit color development, absorbance was read at 718 nm. Standard phosphate curves (0–100 nmol P_i/tube) were conducted in the presence and absence of each addition to check for added phosphate and/or assay interference. (Ca^{2+} + Mg^{2+})-ATPase activity is the increment in basal Mg^{2+}-dependent ATPase due to inclusion of the indicated concentration of free Ca^{2+}. (Na^+ + K^+)-ATPase activity is that measured in the presence of NaCl which can be inhibited by digitoxigenin. ATPase activities were observed to continue linearly under the incubation conditions described above for up to 30 min.

Calcium transport measurement. Ca^{2+} accumulation within synaptic plasma membrane vesicles via the Na^+/Ca^{2+} exchanger (i.e. driven by an outward flux of sodium ions loaded within the vesicles) was measured under conditions similar to those described previously [6,10]. Since the driving Na^+ gradient can be dissipated by the opening of Na^+ channels using the Na^+ channel agonist veratridine [6,9], reduced Na^+ gradient-driven Ca^{2+} accumulation in the presence of veratridine and reversal of this effect with the Na^+-channel blocker, tetrodotoxin, are reflections of the state of Na^+ channels in the membrane vesicles (see Results). 100 μl aliquots of thawed vesicles were Na^+-loaded by diluting the vesicles with 1.4 ml of 150 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) and allowing equilibration in this medium for 20 min at 22°C. After centrifugation (13000 × *g*, 5 min), and rewashing, vesicles were resuspended at 2.22 mg protein/ml, and added to tubes. 10 μl aliquots of the Na^+-loaded vesicles (22 μg) were preincubated for varying times (up to 40 min) in the presence or absence of tetrodotoxin, then rapidly diluted with 200 μl of Na^+-free medium (150 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) with 89.4 μM EGTA-Tris) either with or without 50 μM veratridine. After a 5 min preincubation, 5 μl of 4 mM $CaCl_2$ (containing 18 Ci/mol $^{45}CaCl_2$) was added to give 100 μM total $CaCl_2$ (10 μM free Ca^{2+}), and the incubation continued for 6 min. Ca^{2+} uptake was rapidly terminated by addition of 3 ml of ice-cold 1 mM $LaCl_3$, 150 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), followed by immediate vacuum filtration through 0.22 μm cellulose acetate membrane filters. After a further two washes, filters were dissolved in scintillant and counted.

Materials and miscellaneous procedures. Tris-ATP (vanadate-free), saponin, dithiothreitol, and EGTA were from Sigma. Ionophore A23187 was from Calbiochem-Behring. Ammonium molybdate solution was from Anderson Laboratories. Elon was from Eastman Kodak. Veratridine and tetrodotoxin were generously provided by Drs. Evelyn Grollman and John Daly, NIH, respectively. $^{45}CaCl_2$ was from ICN. Membrane filters (0.22 μm, cellulose acetate, type 11127) were from Sartorius.

Free Ca^{2+} concentrations were carefully controlled using EGTA and computing all complexes between EGTA, ATP, Ca^{2+}, Mg^{2+}, monovalent cations, and protons, as described previously [10] using the stability constants and computer program described by Fabiato and Fabiato [15]. Protein measurements on membrane vesicles were by the method of Lowry et al. [16] against standards of crystalline bovine serum albumin. ATP and EGTA were adjusted to pH 7.4 with Tris before use. The data in each figure or table are derived from single experiments which are representative of at least three separate experiments.

Results and Discussion

The plasma membrane vesicle preparation isolated from purified synaptosomes derived from cerebral cortex, has been shown to exhibit four major flux mechanisms for calcium and sodium ions [3,6,9,10]: an (ATP + Mg^{2+})-dependent Ca^{2+} pump, a Na^+/Ca^{2+} exchanger, an (ATP + K^+)-dependent Na^+ pump, and a veratridine-sensitive Na^+ channel. 'Flux-reversal' studies have established that these four mechanisms coexist within a single population of sealed membrane vesicles [6,9]. Whereas Na^+-Ca^{2+} exchange, Na^+-pump, and Na^+-channel activities are of well established plasma membrane origin, these studies provided the first conclusive evidence for a Ca^{2+}-pump activity being localized within the synaptic plasma membrane [6,9,10].

The studies described in the present report utilize functional characteristics of all four of the above ion translocating mechanisms to investigate the relative orientation of the synaptic plasma

membrane vesicles. Although it is clear from the function of both ATP-dependent Ca^{2+}-pumping [6] and Na^+-pumping activities [9] that a significant proportion of the resealed synaptic plasma membranes exist as inverted vesicles, it has been unclear whether noninverted vesicles coexist within the preparation, and in what relative proportions vesicles of such mixed orientation might be present. In a previous report it was suggested that few noninverted plasma membrane structures were present within the vesicle preparation [9]. However, the concurrence of the two distinct experimental approaches to assess vesicle sidedness, described below, militate against this view and establish the existence of both inverted and noninverted vesicles and also the relative proportions of each within the preparation.

The first category of studies concern ATPase measurements on the vesicles in the presence and absence of the cholesterol-binding plant glycoside, saponin, which selectively permeabilizes cholesterol-rich plasma membranes [10,17,18]. Low concentrations of the the glycoside were recently shown to rapidly permeabilize the plasma membrane vesicles and release Ca^{2+} accumulated via either the Ca^{2+} pump or Na^+/Ca^{2+} exchanger [10]. Whereas our previous reports [6,9,10] described both ATP-dependent Ca^{2+} pumping and Na^+-pumping activities within the synaptic plasma membrane vesicles, these studies did not characterize concomitant ATP hydrolysis associated with their activities. The data in Table I reveal that, in addition to basal Mg^{2+}-dependent ATPase activity, the membrane vesicles exhibit high ATPase activities corresponding with both plasma membrane pumping mechanisms. Thus, the activities of the $(Na^+ + K^+)$-dependent ATPase (digitoxigenin-inhibitable ATPase activity in the presence of both monovalent cations) and $(Ca^{2+} + Mg^{2+})$-dependent ATPase (the activity in the absence of Na^+ stimulated in this case with 10 μM free Ca^{2+}) were 293.9 and 44.5 nmol P_i/mg protein per min, respectively. The values for both activities are close although slightly higher than those reported by others for synaptic [19,20] and axonal [21] plasma membrane fractions. When the ATPase assay was conducted on vesicles in the presence of 0.05% saponin, the activity of the two enzymes increased by 51.0% and 83.6%, respectively. Basal Mg^{2+}-dependent ATPase activity was also increased with saponin, and by a factor (47.4%) close to that of the $(Na^+ + K^+)$-ATPase. At this concentration of saponin, plasma membrane vesicles are fully permeabilized [10,17] and therefore an increment in activity would be expected to result from accessibility gained by substrates to ATPase sites that may have been occluded within sealed, noninverted vesicles. Since 33.8% of total $(Na^+ + K^+)$-ATPase and 32.1% of total Mg^{2+}-ATPase activity are exposed by saponin, this suggests that these values represent the proportion of these activities existing in sealed, noninverted vesicles. Similar reasoning using the $(Ca^{2+} + Mg^{2+})$-ATPase data would predict that 45.5% of this activity is derived from such structures.

An important aspect in determining the validity of the above conclusions is to establish whether saponin only increases the number of exposed ATPase sites as predicted, or whether the glycoside activates ATPase activities by altering the kinetics of the enzyme. With respect to the $(Ca^{2+} + Mg^{2+})$-ATPase, the former assumption appears to hold. Thus as shown in Fig. 1A, the inclusion of 0.05% saponin in the incubation medium results in a consistent increase in activity across a wide range of free Ca^{2+} concentrations. Eadie-Hofstee analysis of the data (Fig. 1B) reveals that the K_m for Ca^{2+} of the $(Ca^{2+} + Mg^{2+})$-ATPase is almost identical under the two conditions (0.13 and 0.12 μM, in the absence and presence of saponin, respectively). On the other hand, the V_{max} of the enzyme in this vesicle preparation increases from 31.5 to 58.5 nmol P_i/mg protein per min, a proportionate increase (86%) similar to that in Table I. This suggests that saponin increases only the number of exposed enzyme sites. The data in Fig. 1 are further significant in revealing virtual identity between the Ca^{2+} affinities of the $(Ca^{2+} + Mg^{2+})$-ATPase and the $(ATP + Mg^{2+})$-dependent Ca^{2+} pump activity in the same vesicles, the K_m of which was recently determined as being 0.11 μM [10].

Upon further reflection, an increment in the $(Ca^{2+} + Mg^{2+})$-ATPase activity in the presence of saponin which exposes the interior of both inverted and noninverted vesicles, could additionally be attributed to a reduction in the accumulation of

Ca^{2+} within inverted vesicles. Since the Ca^{2+} pump is known to rapidly induce a Ca^{2+} gradient across the vesicle membrane in excess of 100-fold [6], it is likely that the reaction rate will be attenuated due to a significant reverse reaction (i.e. Ca^{2+} efflux-mediated ATP formation) as is known to occur for the Ca^{2+} pump in muscle sarcoplasmic reticulum [22]. Therefore, an experiment similar to that in Fig. 1 was undertaken in the presence and absence of the Ca^{2+} ionophore, A23187, which completely abolishes any net Ca^{2+} gradient across the vesicle membrane [6] yet without compromising the integrity of sealed vesicles. The results of such an experiment are shown in Fig. 2. Thus, the inclusion of 10 μM A23187, while having no effect on the K_m of the ATPase for Ca^{2+} (approximately 0.14 μM with or without the ionophore), increases the V_{max} of the reaction by 20.5%, that is, from 36.5 to 44.0 nmol P_i/mg protein per min. Since A23187 and saponin are both equally effective at dissipating the Ca^{2+} gradient arising from the Ca^{2+}-pump activity [6,10], it is concluded that the same fractional increase in $(Ca^{2+} + Mg^{2+})$-ATPase activity results from removal of the constraining Ca^{2+} gradient by either agent. Therefore, it may be assumed that the true unconstrained $(Ca^{2+} + Mg^{2+})$-ATPase activities without saponin in Table I and Fig. 1 are actually 20.5% higher; hence the real proportion of total activity due to exposure of $(Ca^{2+} + Mg^{2+})$-ATPase sites on noninverted vesicles in Table I and Fig. 1 are 34.4% and 35.0%, respectively – values which now closely agree with the corresponding calculations based on the $(Na^+ + K^+)$-ATPase (33.8%) and Mg^{2+}-ATPase (32.1%) measurements described above. The concurrence of these results is further evidence that all three activities reside in the same population of plasma membrane vesicles of which 32–35% exist as non-inverted, sealed structures. In view of the apparently greater relative permeability of the membrane vesicles to Na^+ as opposed to Ca^{2+} [6,9], it is unlikely that Na^+-gradient formation is a significant attenuating factor with respect to $(Na^+ + K^+)$-ATPase activity.

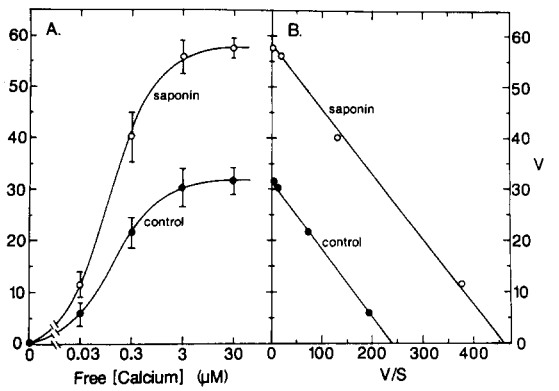
Although the assignment of this value for right-side-out vesicles is fairly conclusive, the above data do not offer information on the remaining population of plasma membranes which may exist as a mixture of noninverted sealed vesicles as well as nonsealed membrane fragments. As stated above, the ATP-dependent Ca^{2+} and Na^+ pumping properties of the vesicles suggest a significant fraction of intact inverted structures. One relatively crude approach to quantitating inverted sealed vesicles was to compare the $(Ca^{2+} + Mg^{2+})$-ATPase activity of nontreated vesicles with their $(ATP + Mg^{2+})$-dependent Ca^{2+} pumping activity, since only the latter is measured within intact

TABLE I

INFLUENCE OF SAPONIN ON $(Na^+ + K^+)$-, $(Ca^{2+} + Mg^{2+})$-, AND Mg^{2+}-DEPENDENT ATPase ACTIVITIES IN SYNAPTIC PLASMA MEMBRANE VESICLES

ATPase assays were run at 37°C for 5 min under the following conditions: $(Na^+ + K^+)$-ATPase activity: 135 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), 2.5 mM ATP-Tris, 100 μM dithiothreitol, either in the presence or absence of 250 μM digitoxigenin; Mg^{2+}-ATPase: 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM EGTA-Tris, 10 mM Tris-HCl (pH 7.4), 2.5 mM ATP-Tris, 100 μM dithiothreitol; $(Ca^{2+} + Mg^{2+})$-ATPase: as for Mg^{2+}-ATPase (that is, in the absence of Ca^{2+}), or in the presence of 200 μM $CaCl_2$ buffered with EGTA to a free Ca^{2+} concentration of 10 μM. Incubations were initiated by addition of 35 μg of synaptic plasma membrane protein in a total volume of 400 μl, and were terminated after 5 min at 37°C by addition of 200 μl of ice-cold 10% trichloroacetic acid. Spectrophotometric determination of inorganic phosphate was as described in Materials and Methods. $(Na^+ + K^+)$-ATPase activity is the difference in activities measured with or without 250 μM digitoxigenin. $(Ca^{2+} + Mg^{2+})$-ATPase activity is the increment in basal Mg^{2+}-ATPase activity with addition of 10 μM free Ca^{2+}. Values are means ± S.D. of three separate determinations measured in duplicate.

ATPase activity	Control activity (without saponin) (nmol P_i/mg per min)	Total activity (with 0.05% saponin) nmol P_i/mg per min)	% Increase due to saponin	Difference as % of total
$(Na^+ + K^+)$-ATPase	293.9 ± 10.6	443.9 ± 9.4	51.0	33.8
$(Ca^{2+} + Mg^{2+})$-ATPase	44.5 ± 4.9	81.7 ± 6.5	83.6	45.5
Mg^{2+}-ATPase	274.9 ± 10.2	405.1 ± 12.7	47.4	32.1



g. 1. Effect of saponin on $(Ca^{2+} + Mg^{2+})$-ATPase activity in naptosomal plasma membrane vesicles. (A) ATPase activity ıs measured at 37°C in 150 mM KCl, 5 mM $MgCl_2$, 10 mM is-HCl (pH 7.4), 2.5 mM ATP-Tris, 100 μM dithiothreitol, her with 0.8 mM EGTA-Tris (for basal Mg^{2+}-dependent TPase) or with 300 μM $CaCl_2$, precisely buffered to the dicated free Ca^{2+} concentrations using EGTA as described rlier [6]. The assays were conducted either in the absence (●) presence (○) of 0.05% saponin. Incubations were initiated addition of 30 μg of synaptic plasma membrane protein in a tal volume of 400 μl, and were terminated after 10 min by dition of 200 μl of 10% trichloroacetic acid, at 0°C. Details the methodology and the spectrophotometric determination inorganic phosphate were as described in Materials and ethods. Basal ATPase in the absence of Ca^{2+} was subtracted)m each value. Each result is the mean ± S.D. of triplicate terminations from one set of experiments. (B) Eadie-Hofstee alysis of the data from (A) which reveals that $(Ca^{2+} + g^{2+})$-ATPase activity in the absence of saponin has a K_m r Ca^{2+} of 0.13 μM, and a V_{max} of 31.5 nmol P_i/mg protein r min, and in the presence of saponin has K_m for Ca^{2+} of 12 μM, and a V_{max} of 58.5 nmol P_i/mg protein per min.

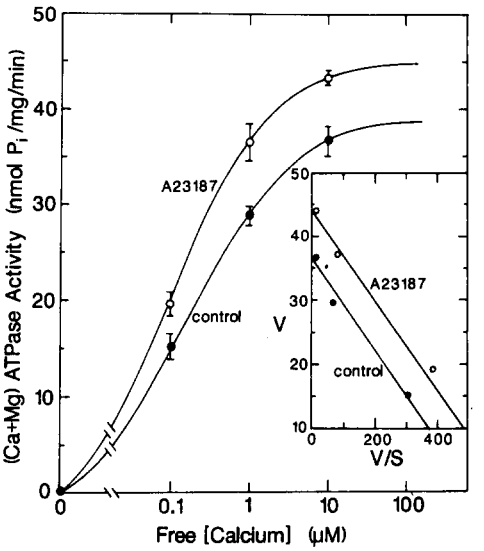


Fig. 2. Influence of Ca^{2+} ionophore A23187 on the $(Ca^{2+} + Mg^{2+})$-ATPase activity of synaptic plasma membrane vesicles. The $(Ca^{2+} + Mg^{2+})$-ATPase assay was in 150 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), 2.5 mM ATP-Tris, 100 μM dithiothreitol, either with 0.3 mM EGTA-Tris (for basal Mg^{2+}-dependent ATPase) without $CaCl_2$, or with 100 μM $CaCl_2$, precisely buffered to the indicated free Ca^{2+} concentrations using EGTA as previously described [6]. ATPase activity was measured either in the absence (●) or presence (○) of 10 μM A23187. The incubations were initiated by addition of 29 μg of synaptic plasma membrane protein in a total volume of 400 μl, and were terminated after 10 min at 37°C by addition of 200 μl of cold 10% trichloroacetic acid. Spectrophotometric determination of inorganic phosphate was as described in Materials and Methods. Basal ATPase in the absence of Ca^{2+} was subtracted from values which are means ± S.D. of triplicate determinations. Eadie-Hofstee analysis of the data (inset) revelas that $(Ca^{2+} + Mg^{2+})$-ATPase activity in the absence or presence of A23187 has a K_m for Ca^{2+} of 0.12 μM, and V_{max} values of 36.5 and 44.0 nmol P_i/mg protein per min in the absence and presence of 10 μM A23187, respectively.

embrane structures. The maximal rate for ATP-pendent Ca^{2+} pumping under the conditions ilized in the ATPase assay is approximately 6–8 nol Ca/mg protein per min, that is, an activity me 5-fold lower than Ca^{2+}-dependent ATPase tivity of the vesicles. Thus, assuming a)ichiometry for the pump of 1 Ca^{2+} ion trans-)rted per ATP molecule hydrolyzed [23], this)uld indicate that only approximately one-fifth the remaining vesicle fraction are intact struc-res. However, there are two problems with at-mpting to relate these two activities. First, al-)ugh the erythocyte Ca^{2+} pump has been re-)rted to have a stoichiometry of 1 [23], the)ichiometry of plasma membrane Ca^{2+} pumping general is unresolved [24]. Second, it is unlikely at Ca^{2+} accumulated within vesicles via the Ca^{2+} pump represents all Ca^{2+} that has been pumped; dissipation of the accumulated Ca^{2+} via, for example, Ca^{2+}-Ca^{2+} exchange [25] mediated perhaps by the Na^+/Ca^{2+} exchanger, is likely to limit Ca^{2+} build-up even though an appreciable Ca^{2+} gradient can be formed [6]. In fact, when Ca^{2+} pumping and Ca^{2+}-ATPase activities are measured at 22°C instead of 37°C, they are differentially reduced by approximately 2.5-fold and 9-fold, respectively (data not shown) indicating a much closer agreement in activities probably as a result of reduced Ca^{2+} leakage at the lower temperature. Indeed, the

only modest Ca^{2+} ionopore-induced increase in Ca^{2+}-ATPase activity in Fig. 2 is testimony to a significant dissipation of accumulated Ca^{2+}, since considerably larger enhancing effects of ionophores can be observed, for example, on the ATPase activity of the purified erythrocyte Ca^{2+} pump reconstituted into nonleaky liposomes [24].

In order to more accurately assess inverted vesicles it was necessary to devise methodology which would explore the relative degrees of orientation of sealed vesicles alone. In a previous report [9], the synaptic plasma membrane vesicles were shown to exhibit several defined Na^+ transport mechanisms included veratridine-dependent Na^+ channels. Using $^{22}Na^+$ to measure Na^+ fluxes, 'flux-reversal' studies revealed that Na^+ channel, Na^+ pump, and Na^+-Ca^{2+} exchange activities all cofunction within the same population of vesicles [9]. This study had indicated that only partial Na^+ channel blockade was achievable with the specific Na^+ channel blocker, tetrodotoxin (TTX). Since tetrodotoxin inhibits channel activity by blockade at a site on the external plasma membrane surface, and since the low hydrophobicity of the molecule precludes its passage across membranes [26], it was probable that the partial action of the antagonist was related to sidedness of the vesicles. However, such studies were difficult to precisely quantitate for several reasons. First, measurement of differences in the rates of passive equilibration of Na^+ ions within vesicles is far more difficult to quantitate and subject to many more nonspecific alterations than measurement of an efficient active transport process, such as the Na^+/Ca^{2+} exchanger or ATP-dependent Ca^{2+} pump, which cause rapid and large accumulations of ions. Second, the vesicles appear to be considerably more permeable to Na^+ ions than, for example, to Ca^{2+} ions, as evidenced by the relatively rapid equilibration and dissipation of Na^+ gradients passively imposed by exposure of the vesicles to high Na^+ concentrations [6]. Third, the specific labeling activity achievable with $^{22}Na^+$ under the required experimental conditions was considerably lower than, for example, that achievable with $^{45}Ca^{2+}$ at low Ca^{2+} concentrations.

Therefore, in order to quantify the differential actions of Na^+ channel-modifiers and assess sidedness of sealed vesicles, a novel alternative strategy was developed to measure Na^+ channel activity. Instead of measuring the passive equilibration of $^{22}Na^+$, Na^+ channel activity was measured by its

TABLE II

DIFFERENTIAL EFFECTS OF VERATRIDINE AND TETRODOTOXIN ON Na^+ CHANNEL ACTIVITY IN SYNAPTIC PLASMA MEMBRANE VESICLES INDIRECTLY MEASURED BY DISSIPATION OF Na^+-GRADIENT DRIVEN Ca^{2+} UPTAKE VIA THE Na^+/Ca^{2+} EXCHANGER

Vesicles were Na^+-loaded by washing and resuspending in 150 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4). After 20 min at 22°C, vesicles were rewashed with the same medium, and 10-μl aliquots of the vesicles (containing 22 μg membrane protein) were preincubated for either 0, 10 or 40 min in the absence or presence of 20 μM tetrodotoxin in the same Na^+-medium. This time period together with the high tetrodotoxin concentration ensured against any time-dependence in the action of tetrodotoxin (see Results). At the end of this period, the vesicles were diluted with 200 μl of Na^+-free medium (150 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) with 89.4 μM EGTA-Tris) either with or without 50 μM veratridine. The vesicles were further incubated for 5 min during which time partial dissipation of the imposed Na^+ gradient occurred which was modified by the presence of veratridine and/or tetrodotoxin (the latter now 1 μM). After exactly 5 minutes, 5 μl of 4 mM $CaCl_2$ (with 18 Ci/mol $^{45}CaCl_2$) was added to give 100 μM $CaCl_2$ (10 μM free Ca^{2+}). Ca^{2+} uptake driven by the remaining Na^+ gradient across the vesicle membrane, proceeded for an additional 6 minutes, at the end of which vesicles were rapidly diluted with 3 ml of ice-cold 1 mM $LaCl_3$, 150 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) followed by immediate vacuum filtration. Results shown are for Ca^{2+} uptake driven by the Na^+/Ca^{2+} exchanger, each value being the mean ± S.D. of triplicate determinations. TTX, tetrodotoxin.

Condition	Na^+-Ca^{2+} exchange-driven Ca^{2+} uptake (nmol/mg protein)		
	0 min preinc	10 min preinc	40 min preinc
Control	1.990 ± 0.036	2.188 ± 0.051	2.266 ± 0.054
Veratridine	1.477 ± 0.014	1.737 ± 0.070	1.794 ± 0.062
TTX	2.088 ± 0.014	2.271 ± 0.010	2.368 ± 0.076
TTX + veratridine	1.746 ± 0.068	1.945 ± 0.041	1.991 ± 0.031

ability to dissipate an imposed Na^+ gradient utilized to drive accumulation of $^{45}Ca^{2+}$ via the Na^+/Ca^{2+} exchanger. This technique had the considerable advantage of using as a signal a large active Ca^{2+} flux in order to measure the passive movement of Na^+ through its chanel. The data in Table II are the results of one set of experiments in which the effects of 50 μM veratridine and 1 μM tetrodotoxin were examined. In this study, vesicles loaded with Na^+ (see Materials and Methods) were respectively preincubated for either 0, 10, or 40 min, in the presence or absence of tetrodotoxin to check for any delayed action of the blocker that might possibly result from its slow penetration within sealed vesicles. The vesicles were then diluted into Na^+-free medium either with or without veratridine, and incubated for a further 5 min. During this period, partial dissipation of the imposed Na^+-gradient took place which was modified by the presence of Na^+ channel effectors. The Na^+ gradient remaining after this period was utilized to drive Na^+-Ca^{2+} exchange-mediated Ca^{2+} uptake after the addition of labeled Ca^{2+} to the reaction vessels for a further 6 min. The large Ca^{2+} uptake so driven thus provides an accurate measurement of the remaining Na^+ gradient, and hence of the degree of induced Na^+ channel-opening.

The data in Table II reveal that the effects of veratridine and tetrodotoxin were highly consistent under the three preincubation conditions. The small increase in Ca^{2+} uptake with the longer preincubation times probably results from slightly higher levels of Na^+ ions loaded within vesicles during the extended preincubation in Na^+ medium. In each case, the Na^+ channel agonist veratridine, induced an almost identical large decrease in Na^+-driven Ca^{2+} uptake due to dissipation of the driving Na^+ gradient resulting from Na^+ channel opening. Since veratridine is lipid-soluble [27] and easily traverses the membrane, it may be assumed that all Na^+ channels are open under this condition, irregardless of vesicle sidedness. The hydrophobic nonpermeant Na^+ channel antagonist tetrodotoxin which interacts only with the external surface of the plasma membrane [26,27], has a slight enhancing effect on Ca^{2+} uptake, which is independent of the time in which tetrodotoxin is preincubated with the vesicles. Under this condition, it is assumed that virtually all Na^+ channels are closed. When experiments are conducted in the presence of both tetrodotoxin and veratridine, there is a consistent partial blockade of veratridine-induced channel opening by tetrodotoxin. Under the three preincubation conditions utilized in the experiments depicted in Table II, this partial blockade by tetrodotoxin accounted, respectively, for 44, 39, and 35% of the veratridine-induced maximal Na^+ channel activation. It is concluded that since tetrodotoxin-blockade is restricted only to noninverted vesicles (inverted vesicles not exposing active sites for tetrodotoxin), this would indicate that between 56 and 65% of the vesicles exist in an inverted state, which is consistent with the measurements of sidedness derived from the ATPase studies described above. Moreover, since the latter studies reveal that 32–35% of total plasma membrane material in the vesicle preparation exists as noninverted vesicles, this suggests that only a small fraction (perhaps less than 10%) of the plasma membranes exist in a nonsealed state. Repeated experimentation similar to that described in Table II did not indicate any consistent alteration of the effect of tetrodotoxin dependent on its preincubation with the vesicles, therefore our previous concern that tetrodotoxin might slowly permeate within sealed vesicles [9] is not substantiated. It is concluded that the greatly enhanced signal size and precision afforded by the above approach to measuring Na^+ channel activity is superior to our previous measurements of this activity [9].

From these studies, it is clear that the synaptic plasma membrane vesicle preparation contains a significant fraction of noninverted sealed structures in addition to the inverted population of vesicles indicated in our previous studies [6,9,10]. Such an assessment of sidedness among the vesicle population has particular importance in view of our recent observation that the Na^+/Ca^{2+} exchanger reveals distinct Ca^{2+} transport sites which saturate in the submicromolar and millimolar Ca^{2+} concentration ranges, respectively, possibly representing sites derived from opposite sides of the plasma membrane [10]. Moreover, the relatively high fraction of inverted vesicles may explain the more substantial quantity of high-affinity Ca^{2+} transport sites associated with this activity as com-

pared to studies on the exchanger in other systems in which the majority of Ca^{2+} transport sites were of lower affinity [28–31]. However, further kinetic assessment of the exchanger is of course necessary before the relative abundance of such sites can be correlated with the degree of sidedness of the vesicles. In addition, separation of vesicles according to orientation is required in order to test whether distinct sites for the Na^{+}/Ca^{2+} exchanger do originate from opposite sides of the membrane. The approaches described in the present report will provide an important means for monitoring such isolation. Furthermore, each of these approaches for measuring vesicle sidedness is highly applicable to studies on membrane preparations derived from a variety of other excitable tissues, including both neural and muscle.

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