ELECTROKINETIC PROPERTIES OF SYNAPTIC VESICLES AND SYNAPTOSOMAL MEMBRANES

D. P. SIEGEL AND B. R. WARE, *Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138 U.S.A.*

ABSTRACT Using the technique of electrophoretic light scattering, we have measured the electrophoretic mobilities of synaptic vesicles and synaptosomal plasma membranes isolated from guinea-pig cerebral cortex. The electrophoretic mobility of synaptic vesicles is slightly greater than that of synaptosomal plasma membranes. Ca^{+2} and Mg^{+2} reduced the mobility of both species to the same extent at physiologically relevant concentrations (0–1 mM) and near-physiological ionic strength. The extent of the reduction was not large (~6% for synaptic vesicles in the presence of 100 mM KCl) at 1 mM divalent cation concentration. At concentrations of ~2 mM and higher, Ca^{+2} reduced the mobility of synaptic vesicles more than did Mg^{+2}. A similar but much smaller effect was observed in the case of synaptosomal plasma membranes. The addition of 1 mM Mg^{+2}-ATP had no effect upon synaptic vesicle mobility, either in the presence or absence of the ionophores nigericin or valinomycin. These data, together with earlier work (Siegel et al., 1978, *Biophys. J.* **22**:341–346), demonstrate that substantial reduction of the average electrostatic surface charge density is not the most important role of divalent cations in promoting close approach of secretory granules and secretory cell membranes, and that it is certainly not the Ca^{+2}-specific step in exocytosis.

INTRODUCTION

It is generally accepted that the secretion of neurotransmitter molecules from nerve terminals occurs via exocytosis of the contents of synaptic vesicles (1). Upon depolarization of the nerve terminal membrane, calcium ions enter from the extracellular medium. This calcium influx stimulates close apposition (virtual contact) and promotes subsequent fusion of the synaptic vesicle membrane with the presynaptic membrane via mechanisms that remain obscure. Magnesium ions will not substitute for calcium ions in stimulating secretion by this process, which occurs in many other secretory systems, such as the adrenal medulla (2).

The electrostatic repulsion of the like-charged secretory vesicle and secretory cell plasma membrane must be overcome to achieve close apposition of the membranes. It is therefore relevant to an understanding of the exocytotic process to consider the possible neutralizing effects of divalent cations upon the electrostatic surface charge density of the membranes of such a system.

Reasonably pure preparations of synaptic vesicles and synaptosomal plasma membranes were first reported in the pioneering work of V. P. Whittaker (3). These techniques and their subsequent refinement by many researchers in more recent years (e.g., 4) have made excellent

---

Dr. Siegel's present address is Miami Valley Laboratories, Proctor and Gamble Co., P.O. Box 39175, Cincinnati, Ohio 45247.

Dr. Ware is an Alfred P. Sloan Research Fellow, and is the author to whom correspondence should be addressed. His present address is Department of Chemistry, Syracuse University, Syracuse, N.Y. 13210.

preparations of these organelles readily achievable. We have undertaken a study of these particles using the technique of electrophoretic light scattering (5–7) in which the measurement of the Doppler shift of scattered light permits a rapid, accurate determination of the electrophoretic mobility of submicroscopic particles, as well as information concerning their state of aggregation.

MATERIALS AND METHODS

Isolation of Synaptic Vesicles

Synaptic vesicles were prepared via a modified version of the shallow gradient technique of Nagy et al. (4), which yields a slightly purer preparation than the standard step-gradient protocol.

All solutions were 5.0 mM morpholino-propane sulfonic acid (MOPS) pH buffer adjusted to pH 7.1 with NaOH and were filtered before use through a well-rinsed 0.2-μm pore-size filter (Dynalab Corp., Rochester, N.Y.). All operations were performed at 0–4°C.

In each of six Beckman-Spinco SW27 rotor centrifuge tubes (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), sucrose density step gradients were formed consisting of 10 ml each of 0.400, 0.300, and 0.200 M sucrose and 8 ml of fraction W_s (the supernatant of the centrifuged lysate of the crude synaptosomal pellet derived from 10 cortices) using a syringe infusion pump (Orion Research Inc. Cambridge, Mass.). These were centrifuged at 20,000 rpm in a Beckman-Spinco L-2 65B centrifuge for 2.5h.

The gradients appeared as clear layers of volume approximately equal to the volume of applied fraction W_s overlying a faintly turbid solution and a small white pellet. The gradients were fractionated with a syringe and a flat-ground needle as follows: the first 8 ml withdrawn from the top of the gradient (the clear layer) was designated fraction 0, the next 5 ml was designated fraction C, the next 10-ml fraction D1, the next 10-ml fraction D2, and the pellet (resuspended in the remaining fluid) designated fraction E-1. This fractionation scheme is somewhat different from that of Nagy et al. (4) for reasons explained below, but uses a similar nomenclature for roughly equivalent fractions. Aliquots of each fraction were set aside and stored at −20°C pending enzyme activity assays, which were generally performed within 2 wk.

Fraction D1 was taken as the source of synaptic vesicles. It was made 100 mM in KCl and ~ 120 mM in sucrose with appropriate stock solutions and pelleted (SW27 rotor, 27,000 rpm for 1.25 h) onto a cushion of 1.20 M sucrose. The resulting vesicle solution was made 0.32 M in sucrose and 27.5 mM in KCl, using an Abbé refractometer (Bausch & Lomb, Rochester, N.Y., model 3-L) and a chloride-specific electrode (Orion Research, Inc.) with appropriate standard solutions. The ionic strength was then 30 mM, including the pH buffer's contribution. Samples for electrophoresis were made up using this stock solution.

Synaptosomal Plasma Membranes

Synaptosomal plasma membranes were isolated according to a slight modification of the technique of Jones and Matus (8). A sample of fraction W_s was made 1.00 M in sucrose using an Abbé refractometer and appropriate stock solutions. 15 ml of this solution was pipetted into the bottom of each of two SW27 rotor tubes. In each tube, 15 ml of 0.833 M sucrose and 8 ml of 0.320 M sucrose were layered on top of it using the syringe pump, as described above. After centrifugation at 20,000 rpm for 2.5 h, the tube was clear from the top down to the 0.833–1.00-M interface, at which a turbid band had appeared. Below this the solution was faintly turbid above a small whitish-brown pellet. Following Jones and Matus (8), the interface was designated the synaptosomal plasma membrane fraction. (The absence of the myelin band in our preparation is probably due to our use of cleaned cortices as starting tissue and our use of a parent fraction greatly depleted of high-sedimentation-coefficient myelin by prior centrifugation at moderate speed.)

The resulting plasma membrane fraction was made 0.320 M in sucrose and 27.5 mM in KCl as above.

Enzyme Assays

Following Whittaker et al. (4) we measured the following marker enzyme activities to assess the purity of our vesicle fraction: lactate dehydrogenase (a soluble cytoplasmic enzyme) by the method described by Kornberg (9); NADPH cytochrome *c* reductase (an endoplasmic reticulum and microsome marker) by the method of Omura and Takesue (0); and acetylcholinesterase (an activity associated with endoplasmic reticulum and plasma membranes of cholinergic neurones) by the method of Ellman et al. (11). Protein was determined by the method of Lowry et al. (12), modified by the prior precipitation of sample with trichloroacetic acid and using bovine serum albumin as a standard. Many workers (e.g., 13–16) have reported a Mg^{+2}-ATPase in synaptic vesicle fractions of mammalian brain. We used this activity as a convenient marker for synaptic vesicles, assaying for it by the method of Kadota et al. (14) modified by addition of 0.1 mM ouabain to inhibit Na^+-K^+ATPase activity.

Another possible Mg^{+2}-ATPase present in this system is mitochondria. We verified, by assay for the mitochondrial marker cytochrome *c* oxidase (17), that mitochondria were wholly confined to the pellet of our density gradient. Moreover, the mitochondrial Mg^{+2}-ATPase inhibitors oligomycin and efrapeptin (A23871) had no effect upon vesicle fraction activity, in accord with Toll and Howard's findings. (15).

Mg-ATPase activity has been shown to be present in very highly purified vesicles from *Torpedo* (18), and the intimate association of this activity with neurotransmitter uptake in mammalian brain synaptic vesicles (15) implies that the activity is intrinsic to the vesicles. The distribution of this activity in our preparation closely resembles the distribution of acetylcholine (an excellent, though difficultly assayed, marker for cholinergic vesicles) in the similar gradients described in reference 5. These observations support the use of Mg^{+2}-ATPase as a synaptic vesicle marker in the present study.

Electrophoretic Light Scattering (Laser Doppler Electrophoresis)

The electrophoretic light-scattering technique and apparatus have been previously described (6,19). In the present work, either a helium-neon laser (632.8 nm wavelength) or an argon-ion laser (operating at 514.5 nm) was used as the coherent light source. The electrophoretic light-scattering chamber was fitted with either platinized-platinum, hydrogen-charged palladium, or silver-silver chloride electrodes. Choice of electrode material was not observed to affect the data.

The electrical conductivity of sample solutions (required for calculation of the applied electric field strength) was determined with a Radiometer CDM-3 conductivity meter (Radiometer, Copenhagen, Denmark) fitted with a thermostatted sample chamber. Viscosities of sample solutions were determined with an Ostwald viscometer in a thermostatted bath.

Samples were stored on ice until electrophoretic measurements were completed (within 36 h of the sacrifice of the animals). All measurements were made at 25°C either in a medium that was 0.320 M in sucrose and 5.0 mM in MOPS (pH 7.1) with sufficient KCl and divalent cation chloride to yield a constant ionic strength of 30 mM (low ionic strength measurements) or in a medium that was 0.150 M in sucrose, 5.0 mM in MOPS, and 100 mM in KCl and various concentrations of Ca^{+2} or Mg^{+2} (high ionic strength measurements).

Samples were degassed at reduced pressure just before use and were injected into the light-scattering chamber through a 0.2-μm pore-size filter (Nuclepore Corp., Pleasanton, Calif.) in the case of synaptic vesicles, or a similar 0.6-μm pore-size filter in the case of synaptosomal plasma membranes.

Reagents

Sucrose (Grade 1), MOPS, and reagents used in enzyme assays were purchased from Sigma Chemical Co. (St. Louis, Mo.). Nigericin was the generous gift of Dr. W. E. Scott of Hoffmann-La Roche, Inc. Efrapeptin was generously donated by Dr. Robert Hamill of Ely Lilly, Inc. All chemicals were of reagent grade.

RESULTS

Purification of Synaptic Vesicles

The distribution of marker enzyme activities in our synaptic vesicle purification density gradient is presented in Table I. Our scheme is slightly different from that of reference 5 in three respects. First, we used a supernatant of the centrifuged crude synaptosomal pellet lysate instead of the total lysate as Whittaker recommends elsewhere (20), which probably results in a lower level of membranous contamination at no cost in vesicle yield. Secondly, we have introduced a fraction "C" between the topmost and synaptic vesicle fraction (fraction D1) because of the slightly higher NADPH-cytochrome *c* reductase activity (indicating light-microsomal contamination) and high cytoplasmic protein levels in that region. Whittaker et al. (6) reported banding of vesicles just below this region at the 0.2–0.3-M interface. We consistently observed a more homogeneous turbidity. However, the distribution of activities in our gradient closely resembles that of reference 5. Thirdly, we omitted the 0.500-M sucrose layer, as we were not interested in fractionation of material in that density region.

The ratios of specific enrichments of vesicle marker to those of contaminant markers are shown in Table II. Specific activity is the activity of an enzyme per milliliter divided by the total concentration of protein in milligrams per milliliters and is therefore a measure of the fraction of total protein represented by that enzyme. Specific enrichment is defined as the specific activity of an enzyme in a particular fraction divided by the specific activity in the parent fraction: in this case, W_s. These data confirm that fraction D1 is the best source of synaptic vesicles from this gradient. The high Mg^{+2}-ATPase activity in the pellet (fraction E-1) was partially reduced by efrapeptin, implying that some of the acitivity was mitochondrial. The residue was probably activity associated with vesicles attached to or entrained by

TABLE I

DISTRIBUTION OF MARKER ENZYME ACTIVITIES IN THE SYNAPTIC VESICLE PURIFICATION GRADIENT EXPRESSED AS PERCENTAGES OF TOTAL RECOVERED ACTIVITY

Fraction	Mg^{+2}-ATPase	AChEase	NCRedase	LDHase	Protein
			(%)		
0	15.0 ± 2.4	23.7 ± 3.8	32.9 ± 7.1	81.0 ± 2.0	52.8 ± 1.8
C	10.6 ± 1.8	4.6 ± 0.5	10.6 ± 0.7	11.6 ± 4.0	11.7 ± 2.8
D1	14.0 ± 1.7	7.0 ± 1.1	11.7 ± 0.7	3.4 ± 1.0	6.7 ± 1.5
D2	5.4 ± 1.2	6.3 ± 1.9	6.3 ± 1.2	1.1 ± 0.5	4.4 ± 0.9
E-1	55.2 ± 2.5	57.8 ± 0.2	38.6 ± 6.0	2.8 ± 1.0	24.4 ± 3.0
Avg. % recovery of activity from from W_s	84	92	100	76	106
			(mg/ml)		
Activity in fraction W_s per gram of tissue	0.34 ± 0.03	0.29 ± 0.14	36 ± 4.3	8.4 ± 1.0	51.8

AChEase is acetylcholinesterase, NCRedase is NADPH cytochrome *c* reductase, and LDHase is lactate dehydrogenase. Indicated errors are average deviations of measurements from four preparations.

Units of activity: Mg^{+2}-ATPase, AChEase, and LDHase; micromoles per minute. NCRedase; nanomoles per minute.

TABLE II
RATIOS OF SPECIFIC ENRICHMENTS OF MARKER ENZYME ACTIVITIES IN SYNAPTIC VESICLE PURIFICATION GRADIENTS

Fraction	SE of Mg^{+2}-ATPase/SE NCRedase	SE Mg^{+2}-ATPase/SE AChEase
0	0.36 ± 0.07	0.62 ± 0.12
C	0.71 ± 0.11	2.1 ± 0.33
D1	0.88 ± 0.06	2.0 ± 0.45
D2	0.58 ± 0.04	0.59 ± 0.04

Abbreviations as in Table I. Indicated errors are average deviations of measurements from four preparations.

sedimenting synaptic membranes and with "coated vesicles" (21). The activity of vesicles associated with the synaptic membrane is thought to be stimulated severalfold (22).

A number of authors (23,24) have reported that some cytoplasmic proteins adsorb to synaptic vesicles under the low-ionic-strength conditions of hypoosmotic lysis, although they desorb at higher ionic strengths. We therefore washed fraction D1 by pelleting it from 100 mM KCl onto a 1.20-M sucrose cushion to remove any such protein and prevent it from altering the surface properties of the synaptic vesicles.

Electrophoretic Mobilities of Synaptic Vesicles

The mean electrophoretic mobility of synaptic vesicles at an ionic strength of 30 mM as a function of Ca^{+2} and Mg^{+2} concentrations is displayed in Fig. 1. The electrophoretic mobilities are negative and are corrected to the values they would assume in a medium with

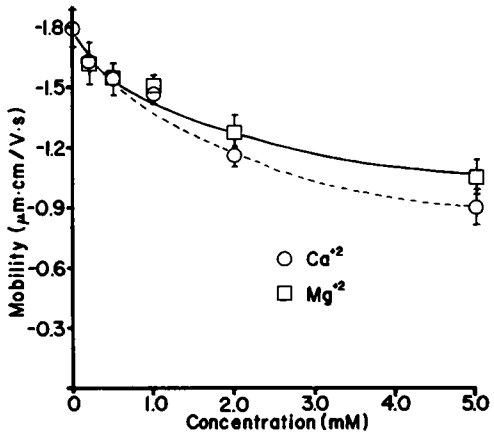

FIGURE 1

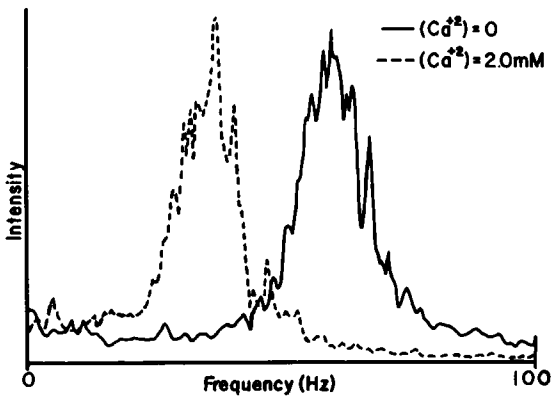

FIGURE 2

FIGURE 1 Electrophoretic mobility of synaptic vesicles as a function of Ca^{+2} and Mg^{+2} concentration at a constant total ionic strength of 30 mM. The mobilities have been corrected to the values they would have in a medium with the viscosity of water at 25°C. The error bars are average deviations of from four to eight sets of measurements.

FIGURE 2 Typical electrophoretic light-scattering spectra of synaptic vesicles in the presence and absence of 2 mM Ca^{+2} at a constant total ionic strength of 30 mM. The horizontal axis is the Doppler shift frequency in hertz, the vertical axis is intensity in arbitrary units. Each of these spectra took ~ 4 min to compile.

the viscosity of water at a temperature of 25°C. The error bars represent the average deviations among from four to eight sets of measurements. A typical pair of electrophoretic light-scattering spectra (taken under the same conditions and total ionic strength, in the presence and absence of 2 mM Ca^{+2}) are displayed in Fig. 2. The horizontal axis is the Doppler shift frequency in hertz, and the vertical axis is intensity in arbitrary units. The Doppler shift is directly proportional to the electrophoretic mobility of the particles.

The widths of such spectra for particles in the size range of subcellular organelles are a function of both the electrophoretic mobility heterogeneity and the diffusive motion of the particles in the sample (19). Extrapolations of plots of half-width at half-height versus electric field strength to zero applied field yield a rough estimate of the weight-average diffusion coefficient of the particles in the sample. This, in turn, yields an approximate estimate of the average diameter, which, in the case of our vesicle samples, is of the order of tenths of micrometers. Although the diameter of synaptic vesicles from mammalian cerebral cortex is smaller than this ($\sim$ 0.05 μm) this result is not surprising: it is in accord with the classical light-scattering measurements of particle size in similar preparations by Formby et al. (25) and with the filtration properties of similar preparations observed by other authors (21,26) even at very low ionic strength, where one would expect aggregation to be less extensive. Moreover, stopped-flow turbidimetry experiments with suspensions of *Torpedo* synaptic vesicles (27) and chromaffin granules (the secretory granules of the adrenal medulla) (28) have demonstrated that these species aggregate readily at moderate univalent salt or millimolar-range divalent cation concentrations. Under our conditions, the product of the Debye-Hückel constant and the particle radius is $> 10^2$. In this regime, the electrophoretic mobility is not sensitive to particle size.

At 30 mM ionic strength, the mobility of the vesicles decreases appreciably as a function of divalent cation concentration from 0 to 2 mM. In this range, Ca^{+2} and Mg^{+2} reduce the mobility of synaptic vesicles to the same extent at each divalent cation concentration. At concentrations of $\sim$ 2 mM and greater, Ca^{+2} reduces the mobility to a greater extent than the same concentration of Mg^{+2}, demonstrating that Ca^{+2} is bound to the vesicle surface to a greater extent than Mg^{+2} at these concentrations. Although the error bars of Ca^{+2} and Mg^{+2} data points overlap slightly, the differences between the two values at these concentrations were consistently of the same sign and similar magnitude in each of six experiments. The state of aggregation at higher Ca^{+2} and Mg^{+2} concentrations was approximately the same as at lower concentrations, although the distribution of electrophoretic mobilities was narrower (as judged from the slope of peak width versus electric field strength plots).

The synaptic vesicle surface affinity for Ca^{+2}, as judged by extent of mobility reduction, was not increased by the presence of Mg^{+2}: the mobility of the vesicles in the presence of 1 mM Ca^{+2} and 1 mM Mg^{+2} was intermediate between the mobilities at 2 mM Ca^{+2} or 2 mM Mg^{+2} only. The state of aggregation underwent no drastic change under these different circumstances.

Most of the measurements of the effects of divalent cations on vesicle mobility were made at an ionic strength of 30 mM, which was experimentally convenient. To determine whether or not the results were markedly different at higher ionic strengths (due, perhaps, to such effects as changes in binding site conformation) three sets of measurements were performed in a medium that was 100 mM in KCl, 150 mM in sucrose (to maintain conditions

approximately isoosmotic to those in the low-ionic strength measurements), 5 mM in MOPS, and at the same pH and temperature. Divalent cations were added as the chlorides from appropriate stock solutions. Because the ionic strength dependence of electrophoretic mobilities is minimal at such high ionic strengths, divalent cations were added without readjustment of the total ionic strength to a constant level. (In no case did the ionic strength vary with divalent cation addition by more than 15%.) The results of these experiments are displayed in Fig. 3. As in Fig. 1, the mobilities are negative and have been corrected to the values they would assume in a medium with the viscosity of water at 25°C. The curves for Ca^{+2} and Mg^{+2} have the same form as the low-ionic-strength data, and exhibit the same Ca^{+2} versus Mg^{+2} selectivity at high concentrations, although the differences in mobility in that region are not as marked. The initial slope of the low-ionic-strength curves is larger than that of Fig. 3. This is probably due in part to the greater effectiveness of divalent cations in electrostatically shielding the surface charge density at highly negative surface potentials than their ionic strength contributions would imply (29). This effect is more noticeable at low ionic strength, where the electrostatic potentials near the surface are more negative (as demonstrated by the more negative electrophoretic mobility) and the divalent cations represent a much higher proportion of the ionic strength due to positive ions than at higher ionic strength.

As in the low-ionic-strength data, the affinity for Ca^{+2} is slightly greater than that for Mg^{+2} at concentrations of 2 mM and higher. The difference between the mobilities at 5 mM Ca^{+2} and 5 mM Mg^{+2} is greater at low ionic strength. This also is probably due in part to the fact that at low ionic strength Ca^{+2} makes up a much larger proportion of the contribution to the total ionic strength made by positive ions than at higher ionic strength (in this case, 57%, vs. 16%). For a given negative surface charge density and bulk Ca^{+2} concentration, the concentration of Ca^{+2} near the surface will be greater at low ionic strength, leading to increased Ca^{+2} binding and a greater proportional reduction of electrophoretic mobility, as observed.

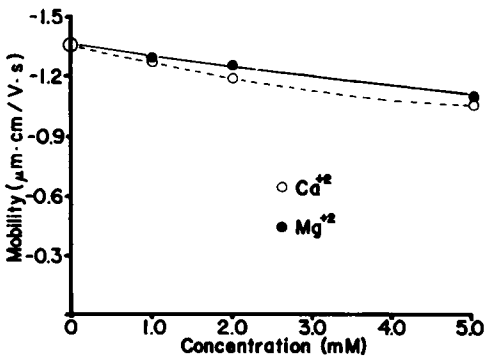

FIGURE 3

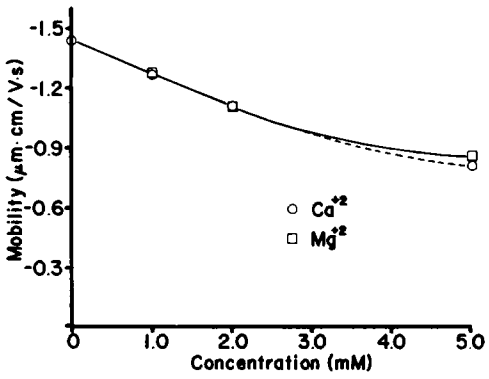

FIGURE 4

FIGURE 3 Electrophoretic mobility of synaptic vesicles as a function of Ca^{+2} and Mg^{+2} concentration in the presence of 100 mM KCl. The mobilities are viscosity-corrected as in Fig. 1. The average deviations are from three sets of measurements, and are contained within the symbols.

FIGURE 4 Electrophoretic mobility of synaptosomal plasma membranes as a function of Ca^{+2} and Mg^{+2} concentration at a constant total ionic strength of 30 mM, viscosity-corrected as in Fig. 1. The average deviations are from three sets of measurements, and are contained within the symbols.

The effects of a number of other reagents were studied at low ionic strength. The synaptic vesicle Mg^{+2}-ATPase is thought to establish a transmembrane gradient in the concentration of H^+ (15,30) as a means of accumulating neurotransmitter molecules and their precursors from the cytoplasm. We therefore attempted to detect changes in the electrophoretic mobility (which might be affected by changes in the transmembrane potential) under circumstances in which the Mg^{+2}-ATPase should be active. In the presence of 1 mMMg^{+2}-ATP the vesicle mobility was virtually unchanged compared with the control value. Furthermore, the addition of the proton ionophore nigericin at a final concentration of 2 μg/ml or of valinomycin at a final concentration of 3 μg/ml in the presence of 1 mM Mg^{+2}-ATP produced no further change in the electrophoretic mobility. Therefore, it seems that the contribution of such a concentration gradient to the electrostatic potential measured via the electrophoretic mobility is undetectably small.

Electrophoretic Mobility of Synaptosomal Plasma Membranes

The electrophoretic mobility of the synaptosomal plasma membrane fraction as a function of Ca^{+2} and Mg^{+2} concentrations at a total ionic strength of 30 mM is presented in Fig. 4. The results are similar to those for synaptic vesicles under the same conditions. In the absence of divalent cations, the mobility of the plasma membranes is 20% less negative than that of the synaptic vesicles. The selectivity for Ca^{+2} vs. Mg^{+2} at higher concentrations is marginal but reproducible.

DISCUSSION

This work establishes that the surface charge density of guinea-pig cerebral cortex synaptic vesicles, as reflected in their electrophoretic mobility, is only a slowly-decreasing function of the concentration of Ca^{+2} and Mg^{+2} in the range 0–1 mM at near-physiological ionic strength. This is the range of Ca^{+2} and Mg^{+2} concentrations thought to be present during stimulus in vivo (31), although there are indications that in some exocytotic systems (e.g., reference 32) much lower levels of intracellular Ca^{+2} are sufficient to induce secretion.

Other authors (33,34), using the technique of optical cytopherometry, have performed electrophoretic measurements on synaptic vesicle suspensions. Microscope electrophoresis is more difficult to apply to the study of particles in this size range, because the particles are difficult to visualize unless highly aggregated, and because the experimenter must select which particles to observe during the measurement. Moreover, compilation of statistically significant samples is laborious, and information concerning the state of aggregation of the particles is not obtained by the measurement. Nevertheless, our measurements at high ionic strength are in close accord with the work of these authors. The value of Matthews and Nordmann (34), obtained in 160 mM KCl, is 2% smaller than ours, probably due in part to the greater ionic strength and correspondingly greater electrostatic shielding of their medium. The value of McLaughlin et al. (33) at 100 mM KCl is the same as ours, -1.36×10^{-4}. At lower ionic strength, McLaughlin et al. report mobilities that are much larger than ours. Although their value was obtained in a different medium (NaCl with 0.3 mM $NaHCO_3$ pH buffer) it is difficult to attribute this discrepancy to the difference in solution conditions, and we are uncertain as to its origin. As these authors note, a value such as they report ($\sim -3.2 \times$

10^{-4} at 30 mM ionic strength) would be much higher than that of any cell or organelle yet reported under the same conditions.

The electrophoretic mobility of synaptosomal plasma membranes is also a slowly-decreasing function of Ca^{+2} and Mg^{+2} concentration. The extent of the electrophoretic mobility reduction of both the synaptic vesicles and plasma membranes at 1 mM Ca^{+2} or Mg^{+2} concentration is small (vesicles: ~ 6% at high ionic strength and 17% at low ionic strength; plasma membranes: 11% at low ionic strength). This indicates that reduction of the average surface charge density of secretory vesicles and secretory cell membranes is unlikely to be the most important role of divalent cations in promoting close apposition of the membranes. That Ca^{+2} and Mg^{+2} reduce the mobilities of these membranes to the same extent in this concentration range demonstrates that such a mechanism is definitely not the Ca^{+2}-specific step in exocytosis.

These results are interesting in light of recent studies of the aggregation of chromaffin granule ghosts (28) and *Torpedo* synaptic vesicles (27). Those authors observed wide-spread aggregation of these species under conditions in which they inferred that only partial neutralization of the repulsive electrostatic forces between them was achieved. They attribute the facile aggregation in these systems to the interaction of proteins that protrude from the secretory granule surface. This interaction becomes possible when limited neutralization of the membranes' mutual electrostatic repulsion has been achieved by divalent cations or electrostatic shielding by higher concentrations of univalent cations. Lateral diffusion in the plane of the membranes would then remove many charged species from the areas of membrane apposed to each other. This rearrangement would permit closer approach of the membranes and the formation of various bridge bonds between them to overcome the effects of electrostatic and short-range hydration forces (35). Substantial reduction of the average surface charge density would thus not be required to promote close approach of secretory granule membranes or, by extension, of granule and secretory cell membranes. Our observations are compatible with this interpretation and its extension to exocytosis. We confirm the limited neutralization of the electrostatic field near the secretory granule surface under the relevant conditions; we observe electrophoretic mobilities of secretory cell membranes that have similar magnitudes and divalent cation dependencies, indicating that extrapolation from granule-granule to granule-cell membrane interactions is possible; and we demonstrate that Ca^{+2} and Mg^{+2} have very similar effects upon the electrophoretic mobility, which is compatible with these workers' findings that Ca^{+2} and Mg^{+2} are of equal effectiveness in aggregating these secretory granules.

Our data also indicate that the synaptic vesicle membranes have a greater affinity for Ca^{+2} than for Mg^{+2} in the 2–5-mM concentration range. Other authors (34) performed microscope electrophoresis measurements of synaptic vesicle mobilities in the presence of 10 mM divalent cations at physiological ionic strength and also observed a greater reduction of the mobility in the presence of Ca^{+2} than in the presence of Mg^{+2}. Hoss et al. (36), through study of the fluorescence of Tb^{+3} bound to the vesicles, have determined that synaptic vesicles from hog and calf brain have a single class of proteinaceous divalent cation binding sites. Further, they determined that the dissociation constants for divalent cation binding to these sites are ~ 2 mM for Ca^{+2} and 5 mM for Mg^{+2}. With the same technique, Haynes et al. (27) determined that similar sites were present in *Torpedo* synaptic vesicles, with competitive inhibition

constants of 1.00 mM for Ca^{+2} and 1.56 mM for Mg^{+2}. Morris and Schober (37) have reported similar sites for chromaffin granules, and have, with electron microscopy, demonstrated that such sites were on the external membranes. These observations describe divalent cation-binding behavior quite similar to that described by our own data and suggest that the effects we observe arise from the same class of sites. It is possible, as Haynes et al. (38) suggest for chromaffin granules, that this class of sites represents the proteinaceous "recognition sites" responsible for the first step in granule-granule and perhaps granule-cell membrane binding.

It has been proposed that selective divalent cation binding to phospholipids is an important step in close apposition and membrane fusion. Portis et al. (39), working with pure dispersions of phosphatidylserine, have determined that Ca^{+2} forms complexes with phosphatidylserine at lower concentrations than Mg^{+2}, that the Ca^{+2} complexes are much more effective at inducing fusion of phosphatidylserine membranes, and that the presence of Mg^{+2} increases the amount of Ca^{+2} complex formed. They speculate that such a mechanism may be involved in the apposition and fusion of membranes in exocytosis. It is not surprising that we failed to observe increased mobility reduction or aggregation in the presence of both ions, however. First, only $\sim$ 15% of the synaptic vesicle phospholipid is negatively charged (40). Secondly, the vesicle population is already significantly aggregated. Finally, the Ca^{+2}-specific complex these workers describe would form between closely-apposed membranes, and would therefore not affect the surface charge density of the membrane area exposed to solution, which is what determines the electrophoretic mobility. Thus, our observations by no means preclude the possibility that Mg^{+2} potentiation of Ca^{+2} binding occurs to some degree: they do establish that it is not a significant electrokinetic effect.

The synaptosomal plasma membranes exhibited marginal selective binding of Ca^{+2} over Mg^{+2} at 5-mM concentration. The divalent cation dependence of the plasma membrane mobility might represent binding of divalent cations to sites with cation binding properties different from those of synaptic vesicles. Alternatively, it might represent binding to a mixture of such sites and a smaller number of sites with Ca^{+2}-selectivity like that of the vesicle sites, perhaps confined to the relatively small area of the synaptosomal plasma membrane that is active towards exocytosis. If this is the case, those sites could be the recognition sites that are complimentary to those of the secretory granules, as suggested by Haynes et al. (38).

It should be noted that the sidedness of these plasma membrane vesicles has not been established. It is conceivable that either the cytoplasmic or extracellular face of the membrane is consistently exposed to the solution in this membrane preparation, or that the preparation is of mixed sidedness. At least one other study (41) of a similar preparation found that the plasma membranes formed resealed vesicles with their original sidedness (extracellular side out). However, other studies of membrane-bound enzyme activities with sidednesses that can be inferred from biochemical criteria (42,43) imply that similar synaptosomal plasma membrane vesicles are either "leaky" or of mixed sidedness. It is therefore impossible to assign the observed electrophoretic properties to either side of the synaptosomal plasma membrane, or even to say with certainty if there should be a difference between the properties of the two. For purposes of comparison, Bosmann's (44) value of the electrophoretic mobility of whole synaptosomes at the same ionic strength as our measurements is -1.43×10^{-4}, very close to our value for synaptosomal plasma membranes of -1.44×10^{-4}.

Creutz et al. have described (45,46) a cytoplasmic protein, synexin, in the adrenal medulla that extensively aggregates chromaffin granules in the presence of submillimolar concentrations of Ca^{+2}; these workers report that their preliminary results indicate the presence of such a protein in brain as well (45). The protein they describe, however, is Ca^{+2} -specific and is saturated with Ca^{+2} at concentrations on the order of 0.3 mM. These are not the divalent cation-binding characteristics displayed by our vesicle preparation, and our preparation is significantly aggregated in the absence of divalent cations. Moreover, we have observed that treatment of the vesicles with either trypsin or papain does not alter the binding preference for Ca^{+2} over Mg^{+2} (47). The aggregating capacity of synexin is trypsin-sensitive (45). We therefore think that our observations are not accounted for by this protein's activity.

We did not observe any effect of Mg^{+2}-ATP upon the mobility of synaptic vesicles, both in the absence and presence of the ionophores nigericin or valinomycin. Previous research (33) also reported a lack of effect of Mg^{+2}-ATP on vesicle mobility. This indicates that the synaptic vesicle Mg^{+2}-ATPase activity has an undetectable influence on the electrokinetic properties of these particles.

The suggestions and encouragement of Dr. David Sattelle and his aid in refining the vesicle preparation are warmly acknowledged. Thanks are due to Ms. Nancy Basore for excellent technical assistance and to Mr. Emil Sefner for expert machining craftsmanship.

Received for publication 11 September 1979 and in revised form 12 December 1979.

REFERENCES

1. LLINAS, R. R. and J. E. HEUSER, editors. 1977. Depolarization-release coupling in neurons. *Neurosci. Res. Program Bull.*, **15**:557–687.
2. DOUGLAS, W. W. 1974. Involvement of calcium in exocytosis and the exocytosisvesiculation sequence. *Biochem. Soc. Symp.* **39**:1–28.
3. WHITTAKER, V. P., I. A. MICHAELSON, R. JEANETTE, and A. KIRKLAND 1964. The separation of synaptic vesicles from nerve ending particles ("synaptosomes"). *Biochem. J.* **20**:293–303.
4. NAGY, A., R. R. BAKER, S. J. MORRIS, and V. P. WHITTAKER. 1976. The preparation and characterization of synaptic vesicles of high purity. *Brain Res.* **109**:285–309.
5. WARE, B. R., and W. H. FLYGARE. 1971. The simultaneous measurement of the electrophoretic mobility and diffusion coefficient in bovine serum albumin solutions by light scattering. *Chem. Phys. Lett.* **12**:81–85.
6. WARE, B. R. 1974. Electrophoretic light scattering. *Adv. Colloid Interface Sci.* **4**:1–44.
7. WARE, B. R. 1978. The study of biological surfaces by electrophoretic light scattering. *In* New Applications of Lasers to Chemistry. Gary M. Hieftje, editor. ACS Symposium Series No. 85. 102–117.
8. JONES, D. H., and A. I. MATUS. 1974. Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. *Biochim. Biophys. Acta.* **356**:276–287.
9. KORNBERG, A. 1955. Lactic dehydrogenase of muscle. *Methods Enzymol.* **1**:1441–443.
10. OMURA, T., and S. TAKESUE. 1970. A new method for simultaneous purification of cytochrome B_5 and NADPH-cytochrome *c* reductase from rat liver microsomes. *J. Biochem. (Tokyo).* **67**:249–257.
11. ELLMAN, G. L., D. COURTNEY, V. ANDRES, JR., and R. FEATHERSTONE. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**:88–95.
12. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
13. HOSIE, R. J. A. 1965. The localization of adenosine triphosphatases in morphologically characterized subcellular fractions of guinea-pig brain. *Biochem. J.* **96**:404–412.
14. KADOTA, K., S. MORI, and R. IMAIZUMI. 1967. The properties of ATPase of synaptic vesicle fraction. *J. Biochem. (Tokyo).* **61**:424–432.
15. TOLL, L., and B. D. HOWARD. 1978. Role of Mg^{+2}-ATPase and a pH gradient in the storage of catecholamines in synaptic vesicles. *Biochemistry.* **17**:2517–2523.

16. TANAKA, R., M. TAKEDA, and M. JAIMOVICH. 1976. Characterization of ATPases of plain and coated vesicle fraction isolated from rat brains. *J. Biochem. (Tokoyo).* **80**:831–837.
17. WHARTON, D. C., and A. TZAGOLOFF. 1967. Cytochrome oxidase from beef heart mitochondria. *Methods Enzymol.* **10**:245–250.
18. ROTHLEIN, J. E., and S. M. PARSONS. 1979. Specificity of association of a Ca^{+2}/Mg^{+2} ATPase with cholinergic synaptic vesicles from *Torpedo* electric organ. *Biochem. Biophys. Res. Commun.* **88**:1069–1076.
19. SMITH, B. A., and B. R. WARE. 1978. Apparatus and methods for laser Doppler electrophoresis. *In* Contemporary Topics in Analytical and Clinical Chemistry, Vol. 2. D. M. Hercules, G. M. Hieftje, L. R. Snyder, and M. A. Evenson, editors. Plenum Publishing Corp., New York. 29–54.
20. WHITTAKER, V. P., and L. A. BARKER. 1972. The sub-cellular fractionation of brain tissue with special reference to the preparation of synaptosomes and their component organelles. *Meth. Neurochem.* **11**:1–52.
21. KADOTA, K., and T. KADOTA. 1973. Isolation of coated vesicles, plain synaptic vesicles, and flocculant material from a crude synaptosomal fraction of guinea-pig whole brain. *J. Cell Biol.* **58**:135–151.
22. BERL, S., S. PUSZKIN, and W. J. NICKLAS. 1973. Actomyosin-like protein in brain. *Science (Wash. D.C.)* **179**:441–446.
23. FONNUM, F. 1967. The "compartmentation" of choline acetyltransferase within the synaptosome. *Biochem. J.* **103**:262–270.
24. WHITTAKER, V. P. 1969. The sub-cellular fractionation of nervous tissue. *In* Structure and Function of Nervous Tissue. Vol. 3. G. H. Borne, editor. Academic Press, Inc., New York. 1–24.
25. FORMBY, B., A. HVIDT, S. J. MORRIS, and J. VIVE LARSON. 1972. The size of rat brain microsomes and synaptic vesicles estimated by light scattering. *Biochim. Biophys. Acta* **279**:407–415.
26. MORGAN, I. G., G. VINCENDON, and G. COMBOS. 1973. Adult rat brain synaptic vesicles. Isolation and characterization. *Biochim. Biophys. Acta.* **320**:671–680.
27. HAYNES, D. H., J. LANSMAN, A. L. CAHILL, and S. J. MORRIS. 1979. Kinetics of cation-induced aggregation of *Torpedo* electric organ synaptic vesicles. *Biochim. Biophys. Acta.* **557**:340–353.
28. MORRIS, S. J., V. C. K. CHIU, and D. H. HAYNES. 1979. Divalent cation-induced aggregation of chromaffin granule membranes. *Memb. Biochem.* **2**:163–201.
29. GRAHAME, D. C. 1953. Diffuse double layer theory for electrolytes of unsymmetrical valence types. *J. Chem. Phys.* **21**:1054–1060.
30. MARON, R., B. I. KANNER, and S. SCHULDINER. 1979. The role of trans-membrane pH gradient in 5-OH-tryptamine uptake by synaptic vesicles from rat brain. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **98**:237–240.
31. PARSEGIAN, V. A. 1977. Considerations in determining the mode of influence of calcium on vesicle-membrane interaction. *Soc. Neurosci. Symp.* **2**:161–171.
32. ZUCKER, R. S., and R. A. STEINHARDT. 1979. Calcium activation of the cortical reaction in sea urchin eggs. *Nature (Lond.).* **279**:820–821.
33. MCLAUGHLIN, J., K. R. CASE, and H. B. BOSMANN. 1973. Electrokinetic properties of isolated cerebral-cortex synaptic vesicles. *Biochem. J.* **136**:919–926.
34. MATTHEWS, E. K., and J. J. NORDMANN. 1976. The synaptic vesicle:calcium ion binding to the vesicle membrane and its modification by drug action. *Mol. Pharmacol.* **12**:778–788.
35. PARSEGIAN, V. A., N. FULLER, and R. P. RAND. 1979. Measured work of deformation and repulsion of lecithin bilayers. *Proc. Natl. Acad. Sci. U.S.A.* **76**:2750–2754.
36. HOSS, W., K. OKUMURA, M. FORMANIAK, and R. TANAKA. 1979. Relation of cation binding sites on synaptic vesicles to opiate action. *Life Sci.* **24**:1003–1010.
37. MORRIS, S. J., and R. SCHOBER. 1977. Demonstration of binding sites for divalent and trivalent ions on the outer surface of chromaffin granule membranes. *Eur. J. Biochem.* **75**:1–12.
38. HAYNES, D. H., M. A. KOLBER, and S. J. MORRIS. 1979. Short and long-range forces involved in cation-induced aggregation of chromaffin granules. *J. Theor. Biol.* **81**:713–744.
39. PORTIS, A., C. NEWTON, W. PANGBORN, and D. PAPAHADJOPOULOS. 1979. Studies of the mechanism of membrane fusion: evidence for an intermembrane calciumphospholipid complex, synergism with magnesium, and inhibition by spectrin. *Biochemistry.* **18**:780–790.
40. WHITTAKER, V. P. 1966. Some properties of synaptic membranes isolated from the central nervous system. *Ann. N.Y. Acad. Sci.* **137**:982–998.
41. CAMPBELL, C. W. B. 1978. The interaction of Cl^-, choline, noradrenaline, and 5-hydroxytryptamine with a purified plasma membrane fraction. *Neurosci. Lett.* **8**:341–346.
42. WELLER, M. 1977. Evidence for the presynaptic location of adenylate cyclase and the cyclic AMP-stimulted protein kinase which is bound to synaptic membranes. *Biochim. Biophys. Acta.* **469**:350–354.

43. SMITH, A. P., and H. H. LOH. 1977. The topographical arrangement of enzymes in synaptosomal plasma membranes. *J. Neurochem.* **29:**1163–1167.
44. BOSMANN, H. B. 1972. Sialic acid on the synaptosome surface and effect of concanavalin A and trypsin on synaptosome electrophoretic mobility. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **22:**97–100.
45. CREUTZ, C. E., C. J. PAZOLES, and H. B. POLLARD. 1978. Isolation and purification of an adrenal medullary protein (synexin) that causes Ca^{+2} -dependent aggregation of isolated chromaffin granules. *J. Biol. Chem.* **253:**2858–2866.
46. CREUTZ, C. E., C. J. PAZOLES, and H. B. POLLARD. 1979. Self-association of synexin in the presence of calcium. *J. Biol. Chem.* **254:**553–558.
47. SIEGEL, D. P. 1979. A study of the membranes of neurosecretory system with laser Doppler electrophoresis. Ph.D. Thesis, Harvard University, Cambridge.