BRIEF COMMUNICATION

THE EFFECTS OF CALCIUM^{2+} AND MAGNESIUM^{2+} ON THE ELECTROPHORETIC MOBILITY OF CHROMAFFIN GRANULES MEASURED BY ELECTROPHORETIC LIGHT SCATTERING

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ABSTRACT Electrophoretic light scattering was used to determine the electrophoretic mobility distributions of isolated bovine adrenal chromaffin granules as a function of divalent metal ion concentrations. Changes in the electrophoretic mobility reflected changes in the surface charge density of the granules. Ca^{2+} and Mg^{2+} (0.10–2.0 mM) were equally effective in reducing the electrophoretic mobilities. These findings are consistent with recent studies of the binding of Ca^{2+} and Mg^{2+} to the surface of chromaffin granules and are further evidence that the specific role of Ca^{2+} in exocytosis is due to effects other than the ability of Ca^{2+} to decrease the electrostatic repulsion between negatively charged membranes.

INTRODUCTION

Chromaffin granules are the catecholamine-containing secretory vesicles found in the chromaffin cells of the adrenal medulla (1). Release of the catecholamines occurs by exocytosis and is mediated by the entrance of Ca^{2+} from the extracellular fluid (2). Mg^{2+} will not substitute for Ca^{2+} in this process (3). The existence of anionic binding sites on the granule membrane has been demonstrated by Eagles et al. (4), using hydroxide and cationized ferritin stains. Studies on the effects of divalent metal cations on the membrane surface properties of the granules have assumed that the information obtained may assist in the elucidation of the mechanism of the granule-granule and/or the granule-membrane interactions that occur during exocytosis.

Banks, using moving boundary electrophoresis, was the first to report that Ca^{2+} decreases the surface charge of the granules (5). Measurements by Matthews et al., and Dean and Matthews, using microelectrophoresis, showed that Ca^{2+} decreases the surface charge of the granules more effectively than Mg^{2+} (6, 7). This latter finding was re-

garded as support for the suggestion that the specific role of Ca^{2+} in exocytosis is to promote fusion of the secretory granule membrane with the plasma membrane by reducing the surface charge density of both species, thereby decreasing their mutual electrostatic repulsion (8).

Aggregation and changes in the ultrastructure of the granules in the presence of Ca^{2+} and Mg^{2+} have been documented by Edwards and co-workers using electron microscopy (9, 10). These findings have been considered to be further evidence that Ca^{2+} may facilitate fusion of membranes by neutralizing their negative surface charge. More recently, however, Morris and Schober (11) have shown that Ca^{2+} and Mg^{2+} are competitive inhibitors of the binding of the fluorescent lanthanide ion Tb^{3+} to the granule membrane and that Ca^{2+} and Mg^{2+} are equally effective in aggregating the granules. The authors raised the possibility that a second Ca^{2+}-specific process may be involved in releasing granule contents once the granule is bound to its specific release site.

We have examined the effect of divalent metal cations on the surface charge of chromaffin granules using the relatively new technique of electrophoretic light scattering (12–14). Electrophoretic mobilities are determined by measuring the Doppler shifts of laser light scattered from the moving particles. For particles in the size range of chromaffin granules, electrophoretic light scattering spectra are broadened both by the diffusion of the particles and by the electrophoretic heterogeneity of the sample. The two effects are easily separated by proper manipulation of experimental variables, so that the size and surface charge of the particles can be inferred from a set of spectra on a single sample.

METHODS

Chromaffin granules were isolated from the bovine adrenal medulla according to the procedure of Trifaro and Dworkind (15) and maintained on ice at a protein concentration of approximately 5 mg/ml. Protein determinations were made by the method of Lowry et al. (16), modified by the prior precipitation of protein with trichloroacetic acid, with bovine serum albumin as a standard. Experiments were performed within 24–40 h after slaughter. Within this period, mobilities remained constant to within 3%.

All light scattering experiments were performed at 20°C in 0.30 M sucrose (5.0 mM PIPES, pH = 6.9) with sufficient concentration of sodium acetate to yield a constant ionic strength of 15 mM. Immediately before the light scattering experiments, the granules were diluted with ice-cold suspension medium. Final protein concentrations of the granules ranged from 2 to 25 μg/ml. Samples were degassed at reduced pressure and injected into the light scattering chamber through a 0.6-μm pore-size hydrophobic Nuclepore filter (Nuclepore Corp., Pleasanton, Calif.).

The electrophoretic light scattering apparatus has been described previously (17). A He-Ne laser (wavelength 632.8 nm) with a 2 mW output was employed. Pulses of constant current were applied to the platinized platinum electrodes of the chamber. The walls of the chamber were coated with methylcellulose to minimize electroosmosis. Solution conductivities (required for the calculation of the electric field strength) were measured with a Radiometer CDM-3 conductivity meter (Radiometer Co., Copenhagen, Denmark). Buffer viscosities were determined with an Ostwald viscometer.

The velocities of the moving granules were calculated from the Doppler-shifted frequencies of the scattered laser light. The electrophoretic mobilities were determined by dividing the velocities by the experimental electric field strength and then correcting to the viscosity of water at 20°C.

Ficoll (type 400) and PIPES buffer were obtained from Sigma Chemical Co. (St. Louis, Mo.). All reagents were of analytical grade.

RESULTS

Representative electrophoretic light scattering spectra of chromaffin granules measured at various concentrations of Ca^{2+} are shown in Fig. 1. The Doppler-shifted frequency of the scattered laser light decreases as the calcium ion concentration is increased. The linewidths were due to a combination of electrophoretic heterogeneity and the spectral broadening due to thermal diffusion.

The electrophoretic mobilities of the granules as a function of the divalent cation concentrations are shown in Fig. 2. Each point in Fig. 2 represents an average of from three to seven measurements. The average deviations for each point are about 2%. To within this precision, Ca^{2+} and Mg^{2+} were equally effective in reducing the electrophoretic mobilities of the granules, with a 50% reduction at about 2 mM. Experiments performed at chromaffin granule concentrations of from 2 to 25 μg protein/ml gave

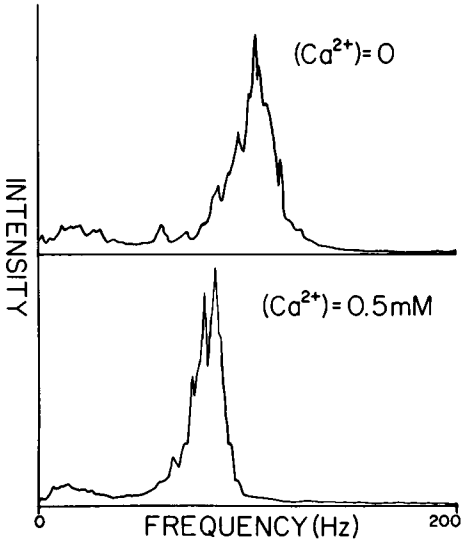

FIGURE 1

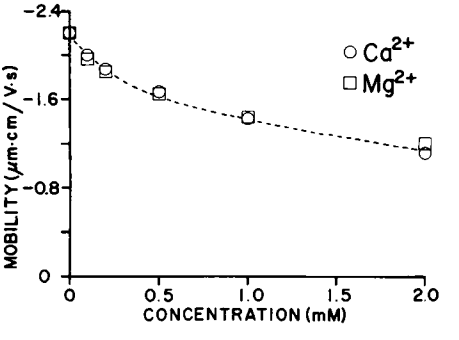

FIGURE 2

FIGURE 1 Representative electrophoretic light scattering spectra of chromaffin granule samples in the absence and presence of 0.50 mM Ca^{2+}. Ordinates are arbitrary.

FIGURE 2 Electrophoretic mobilities of chromaffin granules as a function of Ca^{2+} and Mg^{2+} concentration. Mobilities are negative and corrected to the viscosity of water at 20°C.

the same results. One set of measurements at various concentrations of Ba^{2+} fell on the same curve.

DISCUSSION

Our results demonstrate clearly that chromaffin granules under the solution conditions employed have the same affinity for Ca^{2+} and Mg^{2+}. This finding is in contrast to the reports of Matthews and co-workers (6,7), who found the affinity to be greater for Ca^{2+} than for Mg^{2+}. The differences may be due to differences in the methods of preparation of the granules, the concentrations of the granules, or the solution conditions. The agreement between our results for the fractional reduction of granule electrophoretic mobility in the presence of Ca^{2+} at an ionic strength of 0.015 M and the data of Banks at physiological ionic strength (5) argues against a critical effect of ionic strength on the affinity of the granule membrane for Ca^{2+}. Our results are consistent with the observations of Morris and Schober (11), who measured Ca^{2+} and Mg^{2+} binding to the granules and resultant aggregation of the granules at low ionic strength. The equivalence of Ca^{2+} and Mg^{2+} for reduction of the surface charge of chromaffin granules is evidence that the specific role of Ca^{2+} in exocytosis is due to effects other than the ability of Ca^{2+} to decrease the electrostatic repulsion between charged membranes.

Chromaffin granules are of course a heterogeneous sample, particularly with respect to particle size. The frequency distribution of diameters of isolated chromaffin granules has been determined by Dean (18). The distribution curve has a peak at about 200 nm and a width-at-half-height of about 80 nm. The field-independent component of our light scattering spectra is consistent with this size range. For the ionic strength used in these experiments (0.015 M), the product of the particle radius times the Debye-Hückel constant is about 40, certainly for all particles much greater than one, so the dependence of electrophoretic mobility on particle size is very weak. We do not, therefore, feel that particle size distribution is an important factor in interpreting our results. Of more serious concern is the possibility that cytoplasmic constituents may adsorb to the granule exterior during preparation and thus alter the surface charge and binding properties. Marker enzyme studies in one of our laboratories consistently showed a low degree of contamination (below 5% total) with other membranous material such as mitochondrial, lysosomal, or plasma membrane enzymes.

In principle our spectra could be used to determine the extent of aggregation of the granules by the resultant effect on the diffusion (field-independent) component of the spectral linewidths. However, the variation in these linewidth determinations was too great to permit a precise determination of particle size distribution at each divalent cation concentration. None of the solutions showed any visible evidence of aggregation at any divalent cation concentration. Under very similar conditions, two of us (Westhead and Green), using photon correlation spectroscopy, have shown that threshold concentrations of Ca^{2+} ranging from 2 to 10 mM were required for Ca^{2+}-induced aggregation of chromaffin granules (19).

We have conducted a brief set of similar experiments on plasma membranes isolated from the bovine adrenal medulla. The electrophoretic mobilities of these membrane fractions is about 15% lower than the granule mobilities in the absence of divalent cations. The membrane fraction mobility is also reduced by Ca^{2+} in the concentration range 0.10–2.0 mM by about the same fraction as the mobility of the chromaffin granules. Electrophoretic light scattering spectra of solutions of mixtures of these fractions with chromaffin granules have shown evidence of aggregation in the presence of Ca^{2+}. However, the mobilities of the two fractions become more nearly equal as Ca^{2+} is added, and the electrophoretic resolution is insufficient to obtain quantitative data on specific granule-membrane aggregation. We are currently exploring the potential of the light scattering technique to the study of aggregation in other systems.

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REFERENCES

1. SMITH, A. C., and H. WINKLER. 1972. Fundamental mechanisms in the release of catecholamines. *In* Catecholamines, Handbook of Experimental Pharmacology. H. Blaschko and E. Muscholl, editors. **33**:538–617, Springer Verlag KG, W. Berlin.
2. DOUGLAS, W. W., and R. P. RUBIN. 1963. The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. *J. Physiol. (Lond.).* **167**:288–310.
3. DOUGLAS, W. W. 1974. Involvement of calcium in exocytosis and the exocytosis-vesiculation sequence. *Biochem. Soc. Symp.* **39**:1–28.
4. EAGLES, P. A. M., L. N. JOHNSON, and C. VAN HORN. 1976. The distribution of anionic sites on the surface of the chromaffin granule membrane. *J. Ultrastruct. Res.* **55**:87–95.
5. BANKS, P. 1966. An interaction between chromaffin granules and calcium ions. *Biochem. J.* **101**:18C–20C.
6. MATTHEWS, E. K., R. J. EVANS, and P. M. DEAN. 1972. Electrokinetic properties of isolated chromaffin granules. *Biochem. J.* **130**:825–832.
7. DEAN, P. M., and E. K. MATTHEWS. 1974. Calcium-ion binding to the chromaffin-granule surface. *Biochem. J.* **142**:637–640.
8. DEAN, P. M. 1975. Exocytosis modeling: an electrostatic function for calcium in stimulus-secretion coupling. *J. Theor. Biol.* **54**:289–308.
9. EDWARDS, W., J. H. PHILIPS, and S. J. MORRIS. 1974. Structural changes in chromaffin granules induced by divalent cations. *Biochim. Biophys. Acta.* **356**:164–173.
10. SCHOBER, R., C. NITSCH, and U. RINNE. 1977. Calcium-induced displacement of membrane-associated particles upon aggregation of chromaffin granules. *Science (Wash. D.C.).* **195**:495–497.
11. 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.
12. 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.
13. WARE, B. R. 1974. Electrophoretic light scattering. *Adv. Colloid Interface Sci.* **4**:1–44.
14. FLYGARE, W. H., B. R. WARE, and S. L. HARTFORD. 1976. Electrophoretic light scattering. *In* Molecular Electro-Optics, part I. C. T. O'Konski, editor. Marcel Dekker, Inc., New York. 321–366.
15. TRIFARO, J. M., and J. DWORKIND. 1970. A new and simple method for isolation of adrenal chromaffin granules by means of an isotonic density gradient. *Anal. Biochem.* **34**:403–412.
16. 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.

17. SMITH, B. A., and B. R. WARE. 1978. Apparatus and methods for laser doppler electrophoresis. *In* Current Topics in Analytical and Clinical Chemistry, Vol. II. D. M. Hercules, M. Evanson, G. M. Hieftje, and L. R. Snyder, editors. Plenum Publishing Corp., New York. In press.
18. DEAN, P. M. 1974. Determination of refractive index, mass, and size of secretory particles. *Stud. Biophys.* **43**:227–232.
19. GREEN, D. J., E. W. WESTHEAD, K. H. LANGLEY, and D. B. SATTELLE. 1978. Aggregation and dispersity of isolated chromaffin granules studied by intensity fluctuation spectroscopy. *Biophim. Biophys. Acta.* In press.