MOLECULAR PHARMACOLOGY, 12, 778-788

The Synaptic Vesicle: Calcium Ion Binding to the Vesicle Membrane and Its Modification by Drug Action

E. K. MATTHEWS AND J. J. NORDMANN[1]

Department of Pharmacology, University of Cambridge, Cambridge, England

(Received December 29, 1975)

(Accepted April 19, 1976)

SUMMARY

MATTHEWS, E. K. & NORDMANN, J. J. (1976) The synaptic vesicle: calcium ion binding to the vesicle membrane and its modification by drug action. *Mol. Pharmacol.*, 12, 778-788.

The ionogenic nature of the synaptic vesicle surface and its calcium adsorption characteristics have been investigated by microelectrophoretic measurements on vesicles isolated from the guinea pig cerebral cortex. The electrophoretic mobility of synaptic vesicles determined in 0.16 M KCl was -1.31 μm sec^{-1} cm V^{-1}, corresponding to a surface ζ potential of -18.2 mV and surface charge density of 5451 esu cm^{-2}. Whereas the monovalent cation species of the suspending medium had little effect upon the electrokinetic properties of synaptic vesicles, addition of Ca^{2+}, 1-10 mM, markedly decreased the surface charge. From the monomolecular adsorption isotherm the number of Ca^{2+} binding sites was found to be 4.17×10^{12} cm^{-2}, with an electrochemical free energy of calcium ion adsorption, $\Delta G_{Ca^{2+}}$, of -5.00 kcal mole^{-1}, increasing to -5.64 kcal mole^{-1} at low (0.02) ionic strength. Adenine and pyridine nucleotides (1 mM) were without effect on synaptic vesicle mobility. In contrast, the neuroactive agents trifluoperazine, benzyl alcohol, and amylobarbitone decreased the surface charge of the synaptic vesicle per se, irrespective of any effect on Ca^{2+} binding. These results are discussed in relation to the ionogenic properties of the synaptic vesicle surface and the molecular mechanism of drug action on the neurotransmitter release process.

INTRODUCTION

Normal synaptic transmission in both the central and peripheral nervous systems depends upon a precisely controlled release of neurotransmitter from the presynaptic nerve terminal. Calcium ions are indispensable for the liberation of neurotransmitter evoked by a nerve action potential (1, 2) and appear to play a key role in facilitating the access and adhesion of synaptic vesicles to the terminal membrane for exocytosis (2-4). Various hypotheses have been proposed to account for the critical role of ionic calcium in the stimulus-secretion coupling mechanisms for hormone or neurotransmitter release (5-7), but only recently has consideration been given more explicitly to molecular modification of the physical forces which govern granule- or vesicle-membrane interaction (5, 7-9). Expressed in thermodynamic terms, this involves a decrease, e.g., by Ca^{2+}, of the interfacial potential energy barrier arising from the interplay of electrostatic (i.e., coulombic) and London-van der Waals attractive

[1] Fellow of the European Science Exchange Programme. Present address: Department of Zoology, University of Cambridge.

forces of vesicle and plasma membrane at close approach (5, 10, 11).

Many central depressant drugs, as well as local anesthetic agents, are now believed to exert a major action at nerve terminals by inhibition of the impulse-coupled, calcium-dependent release (i.e., evoked release) of neurotransmitter (12–16). Yet, paradoxically, these and other neuroleptic drugs at the same time markedly *increase* the spontaneous release of transmitter (17, 18). A differential effect of neuroactive agents on the surface charge of synaptic vesicle and presynaptic terminal membrane could account for both, apparently opposing, actions, but little is known either of the ionogenic nature of the two interacting membrane surfaces (9) or of the electrodynamic effects of Ca^{2+} upon them.

It was to determine the characteristics of Ca^{2+} binding to the synaptic vesicle surface, and its stoichiometry, that the experiments reported here were carried out, utilizing the technique of particle microelectrophoresis for an electrokinetic analysis of ion adsorption (8, 19). As an integral part of this study, the effects of a variety of neuroactive agents upon the electrokinetic properties of synaptic vesicles were investigated in an endeavor to resolve the mechanism of action of these agents on the neurotransmitter release process.

Synaptic vesicles isolated from guinea pig brain cerebral cortex were used in this study; the detection and mobility measurement of individual vesicles in the electrophoretic force field was greatly facilitated by incident illumination with a 2.5-mW helium-neon laser.

METHODS

Synaptic vesicle isolation. Synaptic vesicles were obtained from guinea pig cerebral cortex using a modification of the preparative method of Barker, Dowdall, and Whittaker (20). Guinea pigs of either sex, weighing 250–400 g, were fasted for 14 hr. Following decapitation and removal of the superior and inferior colliculi, the cerebral cortex was homogenized in buffered sucrose, 0.32 M (containing Tris-HCl, 5 mM, pH 7.4), to give a 10% (w/v) homogenate. The method of synaptic vesicle isolation differed from the procedure described by Barker *et al.* (20) in that pellet P_2 derived from supernatant S_1 was suspended in water and recentrifuged at 17,000 × *g* for 20 min. The resultant supernatant was combined with pellet P_3 obtained after centrifugation (100,000 × *g* for 20 min in buffered sucrose, 0.32 M) of supernatant fraction S_2. The pooled samples were then placed on a discontinuous sucrose gradient and centrifuged for 2 hr. At the end of that time, fraction D containing synaptic vesicles was isolated, dispersed in buffered sucrose, 0.32 M, and recentrifuged at 100,000 × *g* for a further 60 min. The pellet obtained was then resuspended in 1–2 ml of buffered sucrose, 0.32 M, and maintained at 4° until required for electrophoresis or electron microscopy. As a check of effective separation, dispersions of the various gradient fractions were routinely monitored with a Zeiss UPL stereomicroscope, × 800, under dark-field illumination.

In some experiments mitochondria and synaptosomes were isolated, layers C plus I and B or H of Barker *et al.* (20) corresponding to the mitochondrial and the crude, or disrupted, synaptosomal fractions of the gradient. Each was suspended in buffered sucrose, 0.32 M, and centrifuged at 100,000 × *g* for 60 min. The pellets were then either dispersed in buffered sucrose, 0.32 M, for electrophoresis, or fixed with 5% glutaraldehyde (see below) for electron microscopy.

Microelectrophoresis. The electrokinetic properties of the synaptic vesicles were studied in a microelectrophoretic unit utilizing a thin-walled cylindrical glass cell (mark II, Rank Brothers, Bottisham, Cambs., U. K.) as previously described (8, 19). All measurements of particle electrophoretic velocity were made at the upper, "stationary" level of the cell. A coherent light source was found essential in the present experiments for accurate mobility measurements of synaptic vesicles, which are at the lower limit of detection in white light. The collimated output of a Metrologic 2.5-mW laser (helium-neon, 6328 A) was therefore used for incident (dark-field) illumination of the elec-

trophoretic cell. A heat filter interposed between the laser light source and the electrophoretic cell prevented temperature variation and local convective distortion of the stationary level of the electrophoretic field; the electrophoretic cell was itself immersed in a thermostated water bath maintained at 25° ± 0.1°.

Electrophoretic studies were carried out routinely in 0.16 M KCl. When divalent cations were added to the medium the KCl concentration was correspondingly decreased in order to maintain constant ionic strength. In the studies at low ionic strength sorbitol was used as a substituent to maintain constant osmolarity. The pH of the medium was adjusted to 7.0 with 0.16 M KOH or 0.16 M HCl immediately before addition of the sample dispersion (i.e., synaptic vesicles, mitochondria, or synaptosomes). For the experiments in which neuroactive and other agents were used, the synaptic vesicles were dispersed in the electrophoretic medium (at 25° or 37°) containing the added agent and incubated for 20 min before measurement of vesicle electrophoretic mobility. All electrophoretic measurements were made at 25° ± 0.1° with an applied potential of 40 V. Mean mobilities (± standard errors) were determined for 20 readings, with forward and reverse polarity to minimize electrode polarization and convective effects. Washing the synaptic vesicle preparations in 0.16 M KCl prior to experiment did not change their electrophoretic mobility.

Electron microscopy. For ultrastructural analysis the appropriate gradient layer was dispersed in buffered sucrose, 0.32 M, and centrifuged at 100,000 × g for 60 min. The pellet obtained was resuspended in a buffered glutaraldehyde fixative (5% glutaraldehyde in 0.15 M phosphate solution), and fixation was continued for 60 min at 4°. The samples were then centrifuged at 100,000 × g for 60 min, and the pellets obtained were postfixed with 1% osmic acid for 60 min, followed by dehydration in ethanol, embedding in Epon resin, and sectioning at 750 A with a diamond knife on an LKB ultramicrotome. The sections were placed on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips EM 300 electron microscope.

The diameters of synaptic vesicles, synaptosomes, and mitochondria were calculated by measuring the profile diameter (mean axial ratio) with a circular stencil. It was established that the frequency distribution of synaptic vesicle profile diameters was monomodal (Fig. 1), indicating the preparation of a homogeneous vesicle population. Application of the Giger and Riedwyl transform (see ref. 21) to the data illustrated in Fig. 1 yielded a mean true vesicle diameter of 52 ± 2 nm (n = 301). This may be compared with the mean external diameter of 47 nm (uncorrected for section dimension and therefore an underestimate) calculated by Whittaker, Michaelson, and Kirkland (22) for guinea pig cerebral cortical vesicles.

Theory. In practical electrophoresis the ζ potential of a sphere can be obtained from the Smoluchowski equation:

$$\zeta = \frac{6\pi\eta\mu}{\epsilon f(\kappa\alpha)} \qquad (1)$$

where ζ = ζ potential (millivolts), π = 3.14159, η = bulk viscosity of water (poise), μ = electrophoretic mobility (microns per second per centimeter per volt), ϵ = dielectric constant of water, κ = reciprocal of the thickness of the ionic double layer, α = particle radius (centimeters),

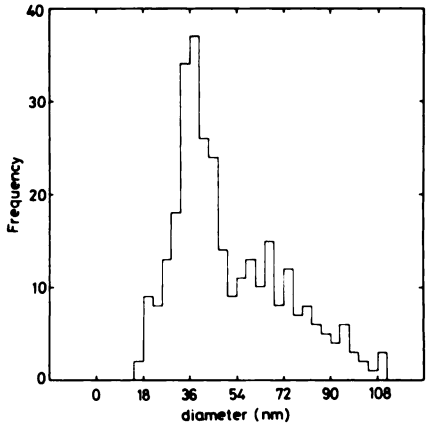

FIG. 1. *Relative frequency distribution of profile diameters of isolated synaptic vesicles*

and $f(\kappa\alpha)$ is a correction factor varying between 1.0 for small κ (i.e., about 0.1) and 1.5 for large κ (i.e., about 1000). For a particle of 50-nm diameter in an aqueous medium at 25° this becomes.

$$\zeta = 13 \cdot 2\ \mu \text{ millivolts} \qquad (2)$$

No corrections are required for ionic relaxation and surface conductance effects provided that the ζ potential is in the vicinity of 25 mV. The surface potential can then be considered equivalent to the ζ potential.

The surface charge density of a particle is related to its electrophoretic mobility by the Gorrin-Gouy equation:

$$\sigma = \left\{\frac{N\epsilon kT}{2000\pi}\sum_{iC_i} \cdot\left[\exp\left(\frac{-1200\ \pi e\eta z_i\ \mu}{\epsilon kT}\right) - 1\right]\right\}^{1/2} \qquad (3)$$

where σ is the surface charge in electrostatic units per square centimeter and has the same sign as μ, defined above [1 esu = $1/(3 \times 10^9)$ coulombs]; N is Avogadro's number; ϵ is the dielectric constant of water at 25°; k is Boltzmann's constant; T is the temperature (absolute); C_i is the molar concentration of ions of the ith species; z_i is the charge on the ions of the ith species with correct sign; η is the viscosity of water at 25° and e is the charge on the electron in electrostatic units.

If counter-ions are adsorbed onto a charged particle surface by a monomolecular process, the subsequent decrease in surface charge, $\Delta\sigma_i$, can be calculated using the equations of Stern as described by Seaman *et al.* (23). Suitable rearrangement of the original equations yields the linear form of the (Langmuir) adsorption isotherm, which for Ca^{2+} as the counter-ion can be expressed as

$$\frac{1}{\Delta\sigma_{Ca^{2+}}} = \frac{1}{2eN_a} + \frac{1}{C_{Ca^{2+}}} \cdot \frac{1}{2eN_aK} \qquad (4)$$

where $\Delta\sigma_{Ca^{2+}}$ is the decrease in charge density of a particle in a solution of C moles per liter of Ca^{2+}; e is the electron charge in electrostatic units; N_a is the number of sites available for adsorption per square centimeter; K = exp. $(\Delta G_{Ca^{2+}} kT)/55.6$, where $\Delta G_{Ca^{2+}}$ is the electrochemical free energy of adsorption of Ca^{2+} in kilocalories per mole and 55.6 is the mole fraction of water; and k and T have been defined above.

Plotting $1/\Delta\sigma_{Ca^{2+}}$ against $1/C_{Ca^{2+}}$ should give a linear regression if the binding of Ca^{2+} to the particle surface is governed by the law of mass action. Values of the number of available Ca^{2+} binding sites at the particle surface and $\Delta G_{Ca^{2+}}$ can be derived from this regression (see ref. 17).

RESULTS

The intraneuronal ionic environment is normally dominated by the concentration of unassociated and diffusible K^+ ions. For this reason a univalent ionic environment of 0.16 M KCl (i.e., ionic strength = 0.16) was chosen for determination of the electrokinetic properties of synaptic vesicles, since it closely approximates the ionic bulk phase of neuronal cytoplasm. This is in contrast to the experiments of McLaughlin, Case, and Bosmann (9), in which a low ionic strength medium of 0.0145 M NaCl was employed for synaptic vesicle mobility measurements with consequential effects on particle charge characteristics (see DISCUSSION). It is clear, nonetheless, from both the microelectrophoretic experiments of McLaughlin *et al.* (9) and our own, that isolated synaptic vesicles possess a high net negative surface charge in comparison with other membrane surfaces of biological origin. Thus the electrophoretic mobility of synaptic vesicles determined in 0.16 M KCl was $-1.31 \pm 0.02\ \mu m\ sec^{-1}\ cm\ V^{-1}$, which corresponds to a surface ζ potential of -18.2 mV and surface charge density of 5451 esu cm^{-2}. The earlier studies of Vos *et al.* (24), using a modified moving boundary technique, and of Ryan *et al.* (25), employing a free-flow electrophoretic method, also recorded a high value for the net negative surface charge of synaptic vesicles, but further comparisons of electrokinetic properties are probably not justified because of the variable viscous drag, ionic strength,

and temperature coefficients inherent in these methods.

As with the vesicle profile diameter distribution (Fig. 1), that for electrophoretic mobility showed a monomodal distribution (Fig. 2). The conclusion that this represents data for a homogeneous population of particles with similar mobilities is strengthened by the fact that the mean mobility values determined for the other particulate fractions were much less (see ratios in Table 1). In these terms the low standard errors of the data included in Table 1 indicate a clear-cut separation of the vesicle population by the preparative method we employed.

Changing the monovalent cation species of the suspending medium had little effect upon the vesicle net negative surface charge, with mobility values of -1.32 ± 0.03 and -1.29 ± 0.04 μm sec^{-1} cm V^{-1} in 0.16 M NaCl and 0.16 M LiCl, respectively. In contrast, the addition of Ca^{2+} ions markedly decreased the surface charge of synaptic vesicles at comparatively low concentrations of added divalent ion, the net negative charge being progressively decreased with increasing Ca^{2+} concentration. Using the Stern (23) model for the adsorption of ions to charged surfaces in aqueous solution, the binding of Ca^{2+} to the synaptic vesicle surface can be calculated, assuming that Ca^{2+} adsorption is governed by the law of mass action (19).

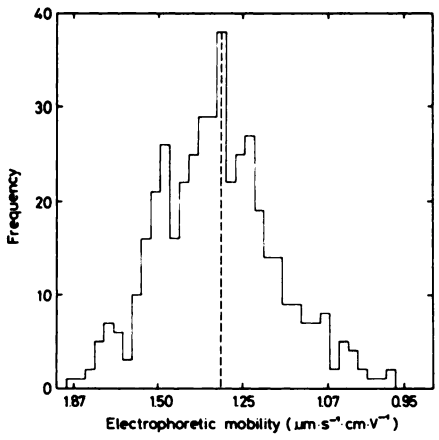

FIG. 2. *Relative frequency distribution of electrophoretic mobility of isolated synaptic vesicles*

Experimental details were the same as for Table 1.

TABLE 1

Electrophoretic mobilities of synaptic vesicles, mitochondria, and synaptosomes from brain cerebral cortex

All electrophoretic measurements were made in 0.16 M KCl at 25° with an applied potential of 40 V. The pH was adjusted to 7.0 with 0.16 M KOH or 0.16 M KCl immediately before addition of the appropriate sample dispersion.

Sample	Mobility	Ratio
	$\mu m\ sec^{-1}\ cm\ V^{-1}$	
A. Synaptic vesicles	-1.31 ± 0.02	A:A, 1.00
B. Mitochondria	-1.04 ± 0.04	B:A, 0.79
C. Synaptosomes	-0.87 ± 0.02	C:A, 0.66
D. Synaptosomes (disrupted)	-0.95 ± 0.03	D:A, 0.73

Thus a double-reciprocal plot of $1/\Delta\sigma_{Ca^{2+}}$ against $1/C_{Ca^{2+}}$ yielded a linear regression, characteristic of the Langmuir adsorption isotherm, with a gradient of 3.0×10^{-6} M esu^{-1} cm^{-2} and an intercept of 2.498×10^{-4} esu^{-1} cm^{-2} (Fig. 3 and Table 2). From these values the number of available calcium binding sites was calculated (from Eq. 4) to be 4.17×10^{12} cm^{-2}, with an electrochemical free energy of calcium ion adsorption, $\Delta G_{Ca^{2+}}$, of -5.00 kcal mole^{-1} (Table 2). These values were obtained at a constant ionic strength of 0.16 M, but since particle electrophoretic mobility is a function of the bulk ionic strength of the suspending medium, some experiments were also carried out at a lower ionic strength of 0.02 to explore the sensitivity of Ca^{2+} binding to the vesicle surface under different electrokinetic conditions, i.e., an increased surface potential and decreased ionization of surface ligands. At an ionic strength of 0.02 the vesicle mobility increased, as expected, to -1.98 ± 0.05 μm sec^{-1} cm V^{-1} from -1.31 ± 0.02 μm sec^{-1} cm V^{-1}, indicative of a higher surface potential, i.e., -27.5 mV as opposed to -18.2 mV. The equilibrium constant for Ca^{2+} binding also increased, from 83.3 to 247.2 M^{-1}, and the electrochemical free energy, $\Delta G_{Ca^{2+}}$, from -5.00 to -5.64 kcal mole^{-1}. The total number of negative binding sites, i.e., surface charge, was apparently decreased at low ionic strength (Table 2), although the percentage of the total negative sites binding

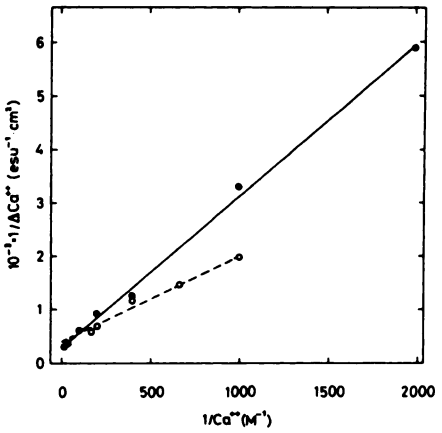

FIG. 3. *Double-reciprocal plot for Ca^{2+} binding to synaptic vesicle surface*

The reciprocal decrease in surface charge caused by the addition of Ca^{2+} ions ($1/\Delta\sigma_{Ca^{2+}}$) is plotted against reciprocal Ca^{2+} concentrations. ●, ionic strength = 0.16; ○, ionic strength = 0.02. Experimental details were the same as for Table 2.

Ca^{2+} was little changed, i.e., 43% and 45% at ionic strengths of 0.16 and 0.02, respectively.

It is possible that adenine and pyridine nucleotides participate in the process of exocytosis (26). There is also evidence that synaptic vesicles possess a ouabain-insensitive, Mg^{2+}-dependent ATPase (27). However, treatment of synaptic vesicles with ATP, 1 mM, at either 37° or 25° in the presence or absence of Mg^{2+}, 1 mM, had no significant effect on vesicle electrophoretic mobility. The pyridine nucleotides NAD and NADPH were similarly without detectable effect.

Effect of neuroactive agents. In the series of psychotropic and local anesthetic agents tested for their effects on the electrokinetic properties of synaptic vesicles, several decreased mobility, and hence surface charge, per se, e.g., amylobarbitone, trifluoperazine, and benzyl alcohol (Table 3). The exceptions were diphenylhydantoin and benzocaine, which had no significant effect on mobility. In contrast, trifluoperazine proved particularly potent, decreasing vesicle mobility markedly at a concentration of 1 μM. In the light of this action, trifluoperazine was tested for its ability to modify the vesicle membrane binding of calcium ions. Figure 4 indicates quite clearly that the decrease in vesicle mobility induced by trifluoperazine is not independent of that induced by Ca^{2+} alone, since on increasing the calcium concentration in the presence of a constant concentration (1 μM) of trifluoperazine, the effect of the anesthetic agent was progressively annulled. The distinction between the two effects was no longer apparent at a concentration of 10 mM Ca^{2+}. Further analysis by a double-reciprocal plot of the data disclosed that the inhibition of membrane calcium binding by trifluoperazine was essentially competitive (Fig. 5).

DISCUSSION

In theory, the surface charge of a membrane-bound particle, e.g., secretory granule or synaptic vesicle, in contact with a polar medium can arise in several ways: from dissociation of surface ionogenic groups, from surface dipole orientation, and from ion adsorption. The char-

TABLE 2

Calcium ion binding to synaptic vesicle surface

Experimental details were the same as for Table 1, except that when divalent cations were added to the medium the KCl concentration was correspondingly decreased to maintain a constant ionic strength (I) of 0.16. In the studies at low ionic strength (0.02) sorbitol was used as a substituent to maintain constant osmolarity. The synaptic vesicles were dispersed in the appropriate Ca^{2+}-containing medium at 25° for 20 min before measurements of electrophoretic mobility were begun.

Adsorption characteristic	I = 0.16	I = 0.02
Intercept (esu^{-1} cm^{-2})	2.498×10^{-4}	3.99×10^{-4}
Gradient (M esu^{-1} cm^{-2})	3.00×10^{-6}	1.62×10^{-6}
Equilibrium constant (K_{eq}) (M^{-1})	83.3	247.2
Electrochemical free energy (kcal mole^{-1})	−5.00	−5.64
Total negative binding sites (cm^{-2})	9.74×10^{12}	5.87×10^{12}
M^{2+} binding sites (cm^{-2})	4.17×10^{12}	2.61×10^{12}
Sites binding M^{2+} (%)	42.8	44.5

TABLE 3

Effect of neuroactive agents on electrophoretic mobility of isolated synaptic vesicles

Experimental details were the same as for Table 1. The synaptic vesicles were dispersed in medium at 37° containing the added agents and incubated for 20 min before measurements of electrophoretic mobility were begun.

Drug	Concentration	No drug	+ Drug	Decrease in mobility caused by drug
	M	$\mu m\ sec^{-1}\ cm\ V^{-1}$		%
Diphenylhydantoin	10^{-4}	1.35 ± 0.02	1.34 ± 0.05	1
Benzocaine	10^{-3}	1.28 ± 0.03	1.25 ± 0.02	2
Trifluoperazine	10^{-7}	1.24 ± 0.03	1.23 ± 0.01	1
	10^{-6}	1.25 ± 0.01	1.09 ± 0.01	13[a]
	10^{-5}	1.28 ± 0.02	1.12 ± 0.02	13[a]
Amylobarbitone	10^{-3}	1.28 ± 0.02	1.19 ± 0.02	7[b]
Benzyl alcohol	10^{-3}	1.29 ± 0.02	1.09 ± 0.02	15[a]

[a] $p < 0.001$.
[b] $p < 0.005$.

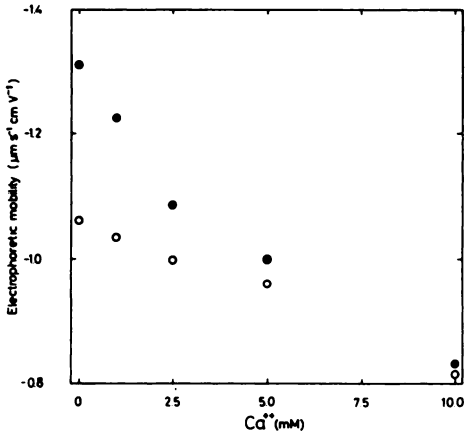

FIG. 4. *Effect of calcium and trifluoperazine on electrophoretic mobility of isolated synaptic vesicles*
●, no trifluoperazine; ○, 1 μM trifluoperazine. Experimental details were the same as for Table 3.

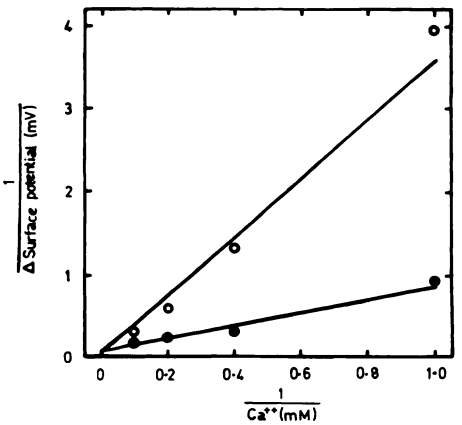

FIG. 5. *Surface potential of isolated synaptic vesicles: interaction of calcium and trifluoperazine*
Double-reciprocal plot of the decrease in surface potential induced by Ca^{2+} in the absence (●) and presence (○) of 1 μM trifluopreazine. Experimental details were the same as for Table 3.

acteristic changes in synaptic vesicle electrophoretic mobility as a function of pH (9) and ionic strength (ref. 9 and present study) specifically exclude a significant contribution from surface dipole orientation and ion adsorption and indicate the major determinant of the vesicle surface charge to be the ionization of surface ligands. From a calculation of surface pK_a values and other evidence, the prototropic dissociation of non-sialic acid carboxyl groups of membrane proteins appears mainly responsible for the high negative surface charge of secretory granules and synaptic vesicles alike (8, 9, 28, 29). The ionization of membrane carboxyl groups will be influenced by the electronegativity of adjacent atoms (see ref. 8). The surface pK_a is thus a function of site field strength (30), which will in turn determine the selectivity pattern for surface adsorption of positively charged counterions to the synaptic vesicle surface. In contrast to chromaffin granules (19), there appeared little difference between the electrophoretic mobility (and hence

surface potential and charge) of synaptic vesicles determined in an aqueous environment of LiCl, NaCl, or KCl at constant ionic strength (0.16). This may indicate either that little distinction exists between the free energy of the univalent cation–surface ligand interaction and the free energy of cation hydration (the cation-H_2O interaction precisely counterbalancing in energetic terms the cation-membrane interaction) or, alternatively, that little specific binding of univalent cations to the negatively charged vesicle membrane occurs. The latter possibility seems the more likely, since in this situation the univalent electrolyte concentration would serve simply to maintain a constant, compressed double layer, with ionic radius and hydration playing a relatively minor role. In marked contrast, divalent cations at low concentration exerted a major influence on synaptic vesicle mobility, the net negative surface charge being progressively decreased with increasing Ca^{2+} concentration.

Although it may be argued that the equilibrium constant for Ca^{2+} binding is only "apparent," in that it contains contributions both from specific adsorption to anionic ligands of the vesicle surface and from screening effects in the electrical double layer, it is likely that the major contribution is derived from a surface binding of calcium, because screening effects are only of major consequence at much higher surface potentials, e.g., above 100 mV (see ref. 31). The double layer is in any case already highly compressed at an ionic strength of 0.16 by the presence of univalent K^+ counter-ions. An 8-fold decrease in ionic strength to 0.02 increases the vesicle surface potential, while the number of negative sites per square centimeter apparently falls from 9.74 to 5.87×10^{12}. This latter effect may represent a decrease in surface ligand ionization or simply indicate the existence behind the shear plane of positive groups, which at normal ionic strength (0.16) are fully screened but at low ionic strength (0.02) are revealed as an apparent negative charge density decrease. Significantly, however, at low ionic strength the percentage of total negative sites binding Ca^{2+} ions remains unchanged, i.e., in the range 43–45%. On the other hand, the free energy of Ca^{2+} binding, which reflects an increased equilibrium constant, is increased from -5.00 to -5.64 kcal mole^{-1}. These results, taken together with the finding that multivalent cations show a definite selectivity pattern for adsorption to secretory granule membranes (8, 9), make it likely that calcium ions are effectively bound to the synaptic vesicle membrane whereas, as pointed out already, the univalent alkali metal cations probably are not.

Secretory granule and synaptic vesicle membranes contain, characteristically, a predominance of Mg^{2+}-ATPase or Ca^{2+}-ATPase activity relative to any (Na^+ + K^+)-ATPase (27, 32). Thus it has been proposed that the Mg^{2+}-ATPase participates in the structural change of synaptic vesicles (33) and chromaffin granules (34) associated with transmitter release. However, it is equally possible that an ATPase activated by divalent cations is of importance for mediating either active uptake of the neurotransmitter or transport of the divalent cation into the vesicle to decrease its local ionized cytoplasmic concentration, without necessarily provoking a major change in membrane structure, because ATP caused no change in the electrokinetic properties of the synaptic vesicle membrane whether in the presence or absence of Ca^{2+} or Mg^{2+}, at 37° or 25°. These results suggest that neither cofactor absence (Mg^{2+}) nor a temperature-sensitive membrane phase transition is of limiting importance in these experiments and indicate quite clearly that if ATPase activation or phosphorylation of vesicle membrane components occurs, it does so without appreciable modification of membrane charge characteristics at the electrokinetic surface of shear. Any change in transmitter release in response to ATPase activation, as recently proposed (26), is therefore more likely attributable to an effect involving primarily the plasma, rather than the vesicle, membrane.

According to the vesicle hypothesis of

transmitter release of Katz (2) as modified by Blioch *et al.* (7), both the synaptic vesicle surface and the inner surface of the prejunctional cell membrane bear net negative charges. In consequence an electrostatic repulsive force exists between the vesicle and cell membrane, which, if it outweighs the London-van der Waals attractive forces, generates a potential energy barrier to membrane adhesion (7). The influx of Ca^{2+} during the action potential (1, 2) may be instrumental in diminishing this barrier, promoting adhesion and thence fusion of the plasma-vesicle membrane and transmitter exocytosis. Although such a mechanism may require further modification to account more precisely for the energetic influence of surface complementarity and site specificity on the adhesion process, the calculation[2] of Table 4 and our results on calcium binding to the synaptic vesicle membrane are nonetheless consistent with an electrodynamic role for Ca^{2+} ions in the *evoked* release of neurotransmitter (10, 11).

An additional mechanism should be considered in the context of our experiments with neuroactive agents, namely, that an increase in spontaneous transmitter release is the outcome of a decrease in the energy barrier but, unlike the evoked release, by a Ca^{2+}-independent mechanism (18). It is improbable that these agents, even if highly surface-active, can cause a significant change in the total attractive force between vesicle and cell membrane, since the van der Waals forces arise primarily from the integration of small component molecular forces over a large surface area, and only come into play at close approach. The two most likely mechanisms are thus a decrease in the electrostatic repulsive force (i.e., surface charge) reducing the potential energy barrier to adhesion, or an increased probability of fusion following adhesion. It is therefore of particular relevance that trifluoperazine, benzyl alcohol, and amylobarbitone all decreased the electrophoretic mobility, and hence the surface charge, of the synaptic vesicle surface, irrespective of any effect on calcium binding. Furthermore, the effect on vesicle mobility is, like that on spontaneous transmitter release (18), unrelated simply to the charge carried by the anesthetic agent, since at pH 7.0 these agents are cationic, neutral, and anionic, respectively. It has been suggested that the increase in spontaneous transmitter release is correlated with the ability of certain (neutral) anesthetics, e.g., ethanol and benzyl alcohol, to increase the membrane binding of calcium (35). Yet other agents, including the phenothiazines, e.g., chlorpromazine, which displace Ca^{2+} from membranes (36, 37) also increase spontaneous transmitter release (13). Our finding of a high potency for trifluoperazine in both decreasing vesicle mobility per se and altering surface calcium binding characteristics may be accounted for by the exceptionally high membrane/solvent

TABLE 4

Topography of anionic and Ca^{2+} binding sites at surface of a synaptic vesicle

Values, derived from the surface charge density, of the total number of electronegative charges distributed over the surface of a synaptic vesicle (mean diameter, 52 nm); the average area surrounding each point charge and the mean separation distance between the anionic sites have been calculated for equal spacing on a two-dimensional lattice in hexagonal array.

	Total anionic sites	Ca^{2+} binding sites
No. of sites	828	354
Area of site (nm^2)	10.26	24.00
Separation between sites (nm)	3.44	5.26
No. of sites on a polar cap 0.18 nm thick[a]	1–4[b]	1–3[b]

[a] The number of ionic sites present on a vesicular polar cap 0.18 nm thick and available for binding to the inner aspect of the cell membrane, i.e., to an essentially planar surface, assuming a Ca^{2+} ionic crystal radius of 0.09 nm.

[b] Minimum to maximum.

[2] These calculations also indicate that in the critical region of the synaptic vesicle surface presented to the neuronal cell membrane the total number of Ca^{2+} binding sites available for promoting adhesion is limited, i.e., $\leqq 3$, unless significant site clustering or relative spatial displacement of the membrane surfaces occurs upon interaction.

partition coefficient (e.g., 1600–1700) of the phenothiazine type compounds (38). This provides an explanation for the influence of trifluoperazine on the vesicle surface charge and divalent cation–surface ligand interaction, in that membrane expansion by insertion of anesthetic molecules may induce a structural perturbation of the vesicle membrane and so alter the stoichiometric number and availability of ionogenic ligands for Ca^{2+} binding. Alternatively, a change in membrane molecular environment may reduce the site field strength and hence the affinity of the binding sites for divalent cations; while raising the environmental Ca^{2+} concentration may progressively annul these effects by displacing the neuroleptic agent from the vesicle membrane, as from the synaptosomal membrane (see refs. 37, 39). Whatever the precise molecular basis for these actions of trifluoperazine, our results have established that neuroactive agents can decrease the net negative charge on synaptic vesicles independently of the charge carried by the drug and independently of any effect it may have on Ca^{2+} binding. Such an effect occurring *in vivo* at either the vesicle membrane, plasma membrane, or both, would, by reducing the energy barrier limiting membrane adhesion,[3] be sufficient to markedly increase the probability of quantal, i.e., spontaneous, transmitter release at the intact synapse. The membrane fluidization inherent in the action of anesthetic and neuroleptic agents may at the same time severely diminish activation of the time- and voltage-dependent channels for Na^+ and Ca^{2+} influx through the neuronal membrane and so prevent the evoked, i.e., synchronized, release of transmitter normally induced by the action potential. Further experiments are obviously required to probe in greater depth the mechanism of drug action on the molecular interplay of vesicle membrane–plasma membrane adhesion and its important sequel, membrane fusion.

[3] For example, it may be calculated from Fig. 2 of ref. 11 that, for particles of 50-nm diameter, a 15% decrease in surface potential (and hence surface charge) of cell and vesicle membrane will approximate to a 40% decrease in the potential energy barrier between them at close approach.

ACKNOWLEDGMENTS

We are indebted to Dr. P. M. Dean and Mrs. G. A. Currell for their expert help in this study.

REFERENCES

1. Katz, B. & Miledi, R. (1965) *Proc. R. Soc. Lond., Ser. B, Biol. Sci.*, **161**, 496–503.
2. Katz, B. (1969) *The Release of Neural Transmitter Substances*, Liverpool University Press, Liverpool.
3. Douglas, W. W. (1968) *Br. J. Pharmacol. Chemother.*, **34**, 451–474.
4. Hubbard, J. I. (1970) *Prog. Biophys. Mol. Biol.*, **21**, 35–124.
5. Matthews, E. K. (1970) in *Calcium and Cellular Function* (Cuthbert, A. W., ed.), p. 163, Macmillan, London.
6. Blaschko, H. F. K. & Smith, A. D. (1971) *Philos. Trans. R. Soc. Lond., Ser. B, Biol. Sci.*, **261**, 273–440.
7. Blioch, Z. L., Glagoleva, I. M., Liberman, E. A. & Nenashev, V. A. (1968) *J. Physiol. (Lond.)*, **199**, 11–35.
8. Matthews, E. K., Evans, R. J. & Dean, P. M. (1972) *Biochem. J.*, **130**, 825–832.
9. McLaughlin, J., Case, K. R. & Bosmann, H. B. (1973) *Biochem. J.*, **136**, 919–926.
10. Dean, P. M. & Matthews, E. K. (1975) *J. Theor. Biol.*, **54**, 309–321.
11. Dean, P. M. (1975) *J. Theor. Biol.*, **54**, 289–308.
12. Seeman, P. (1972) *Pharmacol. Rev.*, **24**, 583–655.
13. Quastel, D. M. J., Hackett, J. T. & Okamoto, K. (1972) *Can. J. Physiol. Pharmacol.*, **50**, 275–284.
14. Blaustein, M. P. & Ector, A. C. (1974) *Mol. Pharmacol.*, **11**, 369–378.
15. Matthews, E. K. & Quilliam, J. P. (1964) *Br. J. Pharmacol. Chemother.*, **22**, 415–440.
16. Quastel, D. M. J., Hackett, J. T. & Okamoto, K. (1972) *Can. J. Physiol. Pharmacol.*, **55**, 279–284.
17. Okada, K. (1967) *Jap. J. Physiol.*, **17**, 245–261.
18. Quastel, D. M. J., Hackett, J. T. & Cooke, J. D. (1970) *Science*, **172**, 1034–1036.
19. Dean, P. M. & Matthews, E. K. (1974) *Biochem. J.*, **142**, 637–640.
20. Barker, L. A., Dowdall, M. J. & Whittaker, V. P. (1972) *Biochem. J.*, **130**, 1063–1080.
21. Weibel, E. R. (1969) *Int. Rev. Cytol.*, **26**, 235–302.
22. Whittaker, V. P., Michaelson, I. A. & Kirkland, R. J. A. (1964) *Biochem. J.*, **90**, 293–303.
23. Seaman, G. V. F., Vasar, P. S. & Kendall, M. J. (1969) *Arch. Biochem. Biophys.*, **135**, 356–362.
24. Vos, J., Kuriyama, K. & Roberts, E. (1968) *Brain Res.*, **9**, 224–230.

25. Ryan, K. J., Kalant, H. & Llewellyn Thomas, E. (1971) *J. Cell Biol.*, **49**, 235–246.
26. Gilbert, J. C., Wyllie, M. G. & Davidson, D. V. (1975) *Nature*, **255**, 237–238.
27. Gilbert, J. C. & Wyllie, M. G. (1976) *Br. J. Pharmacol.*, **56**, 49–57.
28. Dean, P. M. (1974) *Brain Res.* **79**, 397–403.
29. Dean, P. M. (1974) in *Secretory Mechanisms of Exocrine Glands* (Thorn, N. A. & Petersen, O. H., eds.), p. 152, Munksgaard, Copenhagen.
30. Diamond, J. M. & Wright, E. M. (1969) *Annu. Rev. Physiol.*, **31**, 581–646.
31. McLaughlin, S. G. A., Szabo, G. & Eisenman, G. (1971) *J. Gen. Physiol.*, **58**, 667–687.
32. Watson, E. L., Siegel, I. A. & Robinovitch, M. R. (1974) *Experientia*, **30**, 876–877.
33. Matsuda, T., Hata, F. & Yoshida, H. (1968) *Biochim. Biophys. Acta*, **150**, 739–741.
34. Poisner, A. M. & Trifaró, J. M. (1967) *Mol. Pharmacol.*, **3**, 561–571.
35. Seeman, P., Chau, M., Goldberg, M., Sauks, T. & Sax, L. (1971) *Biochim. Biophys. Acta*, **225**, 185–193.
36. Feinstein, M. B. & Paimre, M. (1969) *Fed. Proc.*, **28**, 1643–1648.
37. Roth, S. & Seeman, P. (1972) *Biochim. Biophys. Acta*, **255**, 207–219.
38. Seeman, P., Staiman, A. & Chau-Wong, M. (1974) *J. Pharmacol. Exp. Ther.*, **190**, 123–130.
39. Seeman, P. & Lee, T. (1974) *J. Pharmacol. Exp. Ther.*, **190**, 131–140.