Calcium-Ion Binding to the Chromaffin-Granule Surface

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1. Ca^{2+} ions decreased the surface charge of isolated adrenal-medullary chromaffin granules whether the granules were untreated or previously incubated with neuraminidase.
2. Ca^{2+} binding in both cases followed a Langmuir adsorption isotherm.
3. The chromaffin-granule surface was essentially polyanionic with about 10800 anionic sites per granule, of which 4200 sites are capable of binding Ca^{2+} ions with an electrochemical free energy of adsorption of $-1.32\text{ kJ}\cdot\text{mol}^{-1}$.
4. The surface region of the chromaffin granule was calculated to bind 1306 Ca^{2+} ions at $2.2\text{ mM}\cdot\text{Ca}^{2+}$ (ionic strength $0.16\text{ mol}\cdot\text{litre}^{-1}$).
5. The importance of Ca^{2+} binding to the chromaffin-granule surface is discussed in relation to the hypothesis of secretion by exocytosis.

The catecholamines of the adrenal-medullary chromaffin cell are stored intracellularly within membrane-limited secretory granules. After recognition of an appropriate stimulus by the cell the granules attach to the cell membrane before their contents are discharged. Ca^{2+} ions are vital for the operation of this exocytosis release mechanism (Douglas, 1968), and by a decrease of the interfacial potential-energy barrier may facilitate adhesion of the granule to the cell membrane (Matthews, 1970). Isolated chromaffin granules take up Ca^{2+} ions from solution (Borowitz, 1967), but the final compartmental distribution has not been defined; the extent to which the outer surface of the chromaffin granule binds Ca^{2+} ions is therefore unknown. However, the membrane of the intact chromaffin granule has a net negative charge, and bi- and trivalent cations in the suspending solution are known to be capable of decreasing the surface charge (Banks, 1966; Matthews *et al.*, 1972). Since any decrease in surface charge density caused by a counterion at the electrokinetic surface of a charged particle is related by the Stern (1924) equation to the total number of available counterion-adsorption sites, we have used this relationship to characterize the Ca^{2+} -binding site and to obtain a quantitative assessment of Ca^{2+} -ion adsorption to the isolated chromaffin-granule surface. Changes in surface charge density were determined by measurement of granule electrophoretic mobility (Matthews *et al.*, 1972). In this particular study the method of microelectrophoresis has the important advantage that all measurements are restricted to the electrokinetic surface of shear. In consequence, only events at the granule surface are evaluated and any intragranular uptake of Ca^{2+} ions can be disregarded.

Methods

The medullae were dissected from freshly excised bovine adrenal glands (eight to ten glands for each preparation), blotted dry and weighed. The tissue was minced and homogenized in an all-glass homogenizer in ice-cold sucrose (0.3 M ; 5 ml/g of tissue). A purified chromaffin-granule fraction was isolated from the homogenate by the sucrose-Ficoll- $^2\text{H}_2\text{O}$ centrifugation procedure of Trifaró & Dworkind (1970) as described by Matthews *et al.* (1972).

The electrokinetic properties of the chromaffin granules were studied in a particle microelectrophoresis apparatus as described previously (Matthews *et al.*, 1972). For the Ca^{2+} binding studies the electrophoretic mobilities of the granules were measured in aqueous solutions of CaCl_2 ; KCl was added to maintain a constant ionic strength of $0.16\text{ mol}\cdot\text{litre}^{-1}$. The pH of the solution was adjusted to 7.0 with $0.16\text{ M}\cdot\text{KOH}$ or $0.16\text{ M}\cdot\text{HCl}$ immediately before addition of the granules. Mean mobility values for the granules, by using forward and reverse polarity, were determined from ten readings on four separate preparations of chromaffin granules. To study the effect of anions on surface charge, chromaffin-granule mobility was measured in solutions of NaCl, sodium isethionate, KCl or K_2SO_4 at $I = 0.16\text{ mol}\cdot\text{litre}^{-1}$.

In some experiments the chromaffin granules were incubated at 37°C for 60 min in $0.16\text{ M}\cdot\text{KCl}$, pH 7.0, containing 100 munits of *Vibrio cholerae* neuraminidase/ml (1 unit will liberate $1.0\text{ }\mu\text{mol}$ of *N*-acetylneuraminic acid/min at pH 5.0 at 37°C with *N*-acetylneuraminyllactose as a substrate). The granules were spun down and washed by resuspension in

0.16M-KCl; granule mobility was determined in the presence of different concentrations of Ca^{2+} .

Theoretical

The surface charge of a particle in aqueous solution can be calculated from the electrophoretic mobility of the particle by using the Gorrin-Gouy equation for a solution containing one or more counterions:

$$\sigma = \left\{ \frac{NekT}{2000\pi} \sum_i C_i \left[\exp \left(\frac{-1200\pi e \eta z_i \mu}{\epsilon kT} \right) - 1 \right] \right\}^{\frac{1}{2}} \quad (1)$$

where σ is the surface charge in $\text{esu} \cdot \text{cm}^{-2}$ and has the same sign as μ (1 $\text{esu} = 1/(3 \times 10^9)$ C); N is the Avogadro number; ϵ is the dielectric constant of water at 25°C; k is Boltzman's constant; T is the temperature (°K); C_i is the concentration in $\text{mol} \cdot \text{litre}^{-1}$ of ions of the i th species; Z_i is the charge of the ions of the i th species with correct sign; η is the viscosity of water at 25°C; e is the charge of the electron in esu ; μ is the particle electrophoretic mobility ($\text{cm} \cdot \text{s}^{-1} \cdot \text{V}^{-1} \cdot \text{cm}$).

Stern (1924) constructed a model for the adsorption of ions on to charged surfaces in aqueous solution and deduced an expression for the decrease in surface charge density caused by the adsorption of counterion, which for Ca^{2+} can be expressed as follows:

$$\Delta\sigma_{\text{Ca}^{2+}} = 2en_{\text{Ca}^{2+}} = 1 + \exp \frac{2eN_a}{(-\Delta G_{\text{Ca}^{2+}}/kT)} \chi_{\text{Ca}^{2+}} \quad (2)$$

where $\Delta\sigma_{\text{Ca}^{2+}}$ is the decrease in charge density of a particle in a solution of C $\text{mol} \cdot \text{litre}^{-1}$ of Ca^{2+} ; $\chi_{\text{Ca}^{2+}}$ is the molar fraction of Ca^{2+} where $\chi_{\text{Ca}^{2+}} = C/55.6$; 55.6 is the mole fraction of water; $n_{\text{Ca}^{2+}}$ is the number of Ca^{2+} ions adsorbed per cm^2 ; N_a is the number of sites available for adsorption per cm^2 ; $\Delta G_{\text{Ca}^{2+}}$ is the electrochemical free energy of adsorption of Ca^{2+} in $\text{J} \cdot \text{mol}^{-1}$; k , e and T have been defined above.

If we substitute the expression:

$$\frac{\exp(\Delta G/kT)}{55.6} = K$$

into eqn. (2), rearrangement gives the linear form of the Langmuir adsorption isotherm

$$\frac{1}{\Delta\sigma_{\text{Ca}^{2+}}} = \frac{1}{2eN_a} + \frac{1}{C_{\text{Ca}^{2+}}} \cdot \frac{1}{2eN_a K} \quad (3)$$

Assuming that the binding of Ca^{2+} to the chromaffin-granule surface is governed by the Law of Mass Action then a plot of $1/\Delta\sigma_{\text{Ca}^{2+}}$ against $1/C_{\text{Ca}^{2+}}$ should yield a straight line and values for the number of available Ca^{2+} -binding sites at the granule surface and $\Delta G_{\text{Ca}^{2+}}$ can be calculated. This method has been used successfully for the study of Ca^{2+} binding to erythrocyte surfaces (Seaman *et al.*, 1969).

Results and Discussion

The mobility of bovine adrenal chromaffin granules measured in 0.16M-KCl at pH 7 was $-1.077 \mu\text{m} \cdot \text{s}^{-1} \cdot \text{cm} \cdot \text{V}^{-1}$, corresponding to a surface potential of -13.9 mV and surface charge density of $3825 \text{ esu} \cdot \text{cm}^{-2}$. Incubation of chromaffin granules with neuraminidase decreased the mobility to $-0.88 \mu\text{m} \cdot \text{s}^{-1} \cdot \text{cm} \cdot \text{V}^{-1}$, whereas granules incubated at 37°C without neuraminidase treatment had mobilities of $-1.00 \mu\text{m} \cdot \text{s}^{-1} \cdot \text{cm} \cdot \text{V}^{-1}$. Neuraminidase treatment decreased the surface potential to -11.3 mV and the surface charge density to $3005 \text{ esu} \cdot \text{cm}^{-2}$. Both untreated and neuraminidase-treated chromaffin granules were exposed to solutions of different Ca^{2+} concentration and the corresponding surface charge density was measured and plotted in Fig. 1. Although the two curves are of similar shape, the surface charge density for neuraminidase-treated granules was less at all Ca^{2+} concentrations. The surface charge was neutralized at 50mM- Ca^{2+} and 25mM- Ca^{2+} on normal and neuraminidase-treated granules respectively.

A plot of $1/\Delta\sigma_{\text{Ca}^{2+}}$ against $1/C_{\text{Ca}^{2+}}$ for normal chromaffin granules and neuraminidase-treated granules produced straight-line regressions (Fig. 2), suggesting that the adsorption of Ca^{2+} on to the electrokinetic surface occurred as predicted by the Langmuir adsorption isotherm. For untreated chromaffin granules the intercept and slope had

Fig. 1. Effects of Ca^{2+} ions on the surface charge density of untreated and neuraminidase-treated chromaffin granules

Chromaffin granules were previously incubated in *Vibrio cholerae* neuraminidase for 60min before surface-charge measurements were made in solutions of ionic strength 0.16 $\text{mol} \cdot \text{litre}^{-1}$ (○). Granules not treated with neuraminidase served as controls (●). The lines labelled 'Control' and 'After neuraminidase' are the values for surface charge density in 0.16M-KCl alone.

values of $3.37 \times 10^{-4} \text{esu}^{-1} \cdot \text{cm}^2$ and $1.65 \times 10^{-6} \text{esu}^{-1} \cdot \text{cm}^2 \cdot \text{mol} \cdot \text{litre}^{-1}$ and for neuraminidase-treated granules 4.42×10^{-4} and 1.34×10^{-6} respectively. By substituting these values into eqn. (3) the number of Ca^{2+} -binding sites can be derived: $3.08 \times 10^{12}/\text{cm}^2$ of normal chromaffin-granule surface and 2.35×10^{12} binding sites/ cm^2 of neuraminidase-treated granule surface. From the value of the constant K the electrochemical free energy of adsorption of Ca^{2+} , $\Delta G_{\text{Ca}^{2+}}$ was calculated to be $-1.32 \text{kJ} \cdot \text{mol}^{-1}$ and $-1.39 \text{kJ} \cdot \text{mol}^{-1}$ for normal and neuraminidase-treated granules respectively.

To test for the presence of positive ionogenic groups on the surface of the granule membrane, the effects of different anions on the mobility of normal chromaffin granules were examined; the results are shown in Table 1. The anionic species Cl^- , SO_4^{2-} or isethionate were without effect on chromaffin-granule mobility, whereas the counterion Na^+ decreased mobility when compared with measurements made in solutions containing K^+ as the counterion.

It is known that adrenal-medullary chromaffin granules have a net negative charge at the electrokinetic surface (Banks, 1966; Matthews, 1971; Matthews *et al.*, 1972). Certain bi- and ter-valent cations decrease the surface charge, and with La^{3+} charge reversal occurs (Matthews *et al.*, 1972). In the present study it has been observed that Na^+ ions have a greater effect in decreasing granule mobility than K^+ ions. If the granule possesses major positive ionogenic groupings then solutions of different anions should produce an increase in granule negative mobility by binding to the available positive

Table 1. *Electrophoretic mobilities of chromaffin granules in solutions of different ions at pH 7.0*

The ionic strength of the solutions was $0.16 \text{mol} \cdot \text{litre}^{-1}$. The values are given as means $\pm$ S.E.M. for four experiments. Further details are given in the text.

Ionic species	Mobility ($\mu\text{m} \cdot \text{s}^{-1} \cdot \text{cm} \cdot \text{V}^{-1}$)
NaCl	0.881 ± 0.066
Sodium isethionate	0.882 ± 0.107
KCl	1.097 ± 0.090
K_2SO_4	1.096 ± 0.071

sites. However, no difference in effect on mobility was found between the bivalent anion SO_4^{2-} , the bulky univalent anion isethionate, or Cl^- ions. This lack of an anionic 'spectrum' suggested that there exists at pH 7.0 and at an ionic strength of $0.16 \text{mol} \cdot \text{litre}^{-1}$ no significant population of positive groups in the charge-determining region of the chromaffin-granule membrane. Therefore the net negative charge at the granule surface is probably related directly to the total number of anionogenic sites, and the surface can be regarded as essentially polyanionic. This corresponds to previous findings for the erythrocyte membrane, which has no positively charged groups at its surface contributing significantly to the surface potential (Heard & Seaman, 1960; Seaman & Heard, 1960).

Ca^{2+} ions decreased the surface charge of chromaffin granules. The relationship between the decrease in surface charge density and Ca^{2+} concentration was found to fit a Langmuir adsorption isotherm from which could be derived the number of surface adsorption sites for Ca^{2+} ions, i.e. 4206 sites. Pretreatment of the chromaffin granules with neuraminidase removed a small proportion of the total negative charge, namely 21.4% of the total anionic sites and a similar fraction, 23.6% of Ca^{2+} -adsorption sites. This fraction of sites removed by neuraminidase treatment is likely to be an overestimate since incubation at 37°C in the absence of neuraminidase could have accounted for about 40% of the decrease in electrophoretic mobility. Neuraminidase cleaves neuraminic acid from mucopolysaccharides (Gottschalk, 1960) and our previous results showed that this enzyme successfully removed all the neuraminic acid from the chromaffin-granule membrane (Matthews *et al.*, 1972). Evidently, therefore, the ionized carboxyl groups of neuraminic acid account for only a small proportion of the total anionic and Ca^{2+} -binding sites of the chromaffin granule membrane, i.e. about 20%. This may be contrasted with the erythrocyte membrane where Seaman *et al.* (1969) found that for Ca^{2+} binding to the erythrocyte surface, 65% of the total anionic

Fig. 2. *Double-reciprocal plot for Ca^{2+} binding to the chromaffin-granule surface*

The reciprocal decrease in surface charge caused by the addition of Ca^{2+} ions ($1/\Delta\sigma_{\text{Ca}}$) is plotted against the reciprocal Ca^{2+} concentration. ●, Untreated chromaffin granules; ○, neuraminidase-treated granules.

Table 2. *Spatial characteristics of anionic and Ca^{2+} -binding sites at the surface of a chromaffin granule (mean diameter 208.5 nm)*

Details of the calculation are given in the text.

	Total anionic sites	Ca^{2+} -binding sites
Number of sites	10871	4206
Area of site (nm^2)	12.55	32.41
Separation between sites (nm)	3.80	6.11

sites bound Ca^{2+} and that 50% of the anionic charge was removed by neuraminidase together with 78% of the Ca^{2+} -binding sites. In addition to this difference in the relative contribution of neuraminic acid to the total anionic and Ca^{2+} -liganding capacity of the two membranes, Ca^{2+} ions are more strongly bound to the chromaffin-granule surface than the erythrocyte membrane, the electrochemical free energies of Ca^{2+} binding being -1.32 and $-0.91 \text{ kJ} \cdot \text{mol}^{-1}$ respectively.

Using a ^{45}Ca -uptake method, Borowitz (1967) showed that chromaffin granules take up about $16 \mu\text{mol}$ of Ca^{2+} /g of protein in a solution of 2.2 mM - Ca^{2+} and 0.15 M -KCl. This value represents the total uptake of Ca^{2+} . From our experiments we can calculate the amount of Ca^{2+} bound to the surface alone, since the electrokinetic measurements apply only to the critical outermost region. The protein content of wet adrenal-medullary chromaffin granules is 11.5% (Hillarp, 1960). The granules isolated in our study have a mean diameter of 208.5 nm and a wet mass of 5.63 fg (Dean, 1974). From the slope and intercept value of the adsorption curve for Ca^{2+} , the number of Ca^{2+} ions bound per cm^2 of granule surface is calculated to be 95.7×10^{10} ions at a solution concentration of 2.2 mM - Ca^{2+} (1306 Ca^{2+} ions/granule), which can be expressed as $3.35 \mu\text{mol}$ of Ca^{2+} /g of protein; this quantity is bound to the surface. Therefore of the total uptake of Ca^{2+} ions by chromaffin granules we might expect that 20% would be bound to the outer surface of the granule.

From the value of surface charge density it is possible to calculate the total number of electro-negative charges distributed over the granule surface, the average area surrounding each point charge, and the mean separation distance (for a hexagonal array) between the anionic sites (Table 2).

It has been suggested that Ca^{2+} ions may bind to carboxyl groupings at the secretory granule surface and act as a bridge between the granule and the

membrane (see Matthews, 1970). If we consider the chromaffin granule to be a rigid sphere of diameter 208.5 nm and the Ca^{2+} ionic crystal radius to be 0.09 nm we can estimate how many ionic sites would be present on the polar cap 0.18 nm in thickness which would be available for bonding to the inner aspect of the cell membrane, i.e. to an essentially planar surface. From this calculation ten electro-negative sites, of which four may be Ca^{2+} -binding sites, would be present in the polar region as defined. The number of surface Ca^{2+} sites occupied at any one time is obviously proportional to the environmental Ca^{2+} concentration. For example, at a concentration of 2.5 mM, theoretically 1.62 Ca^{2+} -binding sites would be occupied on average, which from geometrical considerations of an integral number of sites would allow between one and three Ca^{2+} sites to be occupied, in that critical region of the granule surface presented to the cell membrane. Thus on this basis, the number of Ca^{2+} sites actually available for promoting granule adhesion before exocytosis is obviously limited, i.e. less than 4, unless significant clustering occurs.

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