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STRUCTURAL CHANGES IN CHROMAFFIN GRANULES INDUCED BY DIVALENT CATIONS

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SUMMARY

Isolated chromaffin granules aggregate reversibly in the presence of Ca^{2+} and Mg^{2+}. The adhering membranes maximise their areas of contact, leading to distortions of the granules and, in many cases, to membrane rupture. The adhesion is accompanied by changes in the structure of the core: it is seen to withdraw from regions of the membrane that are involved in adhesion to other granules, although it remains in contact with the membrane at other places. At the same time the osmotic pressure within the granules increases and their soluble components are released to the medium. These processes can occur at 0 °C, and at low pH, as well as at room temperature and pH 7. Similar reorganisation of membranes and cores may occur during the process of Ca^{2+}-dependent exocytosis in secretion.

INTRODUCTION

The cells of the adrenal medulla (known as 'chromaffin' cells) are packed with membrane-bound secretory granules. The cores of these granules contain either adrenalin or noradrenalin, in conjunction with ATP and protein, at very high concentrations (estimated to be, respectively, about 0.5 M, 0.125 M and 120 mg/ml within the granules [1]). Depolarisation of the cell leads to release of the contents of one or more chromaffin granule(s) by Ca^{2+}-dependent exocytosis [2, 3]. This process, involving fusion of the granule membrane with the plasma membrane, is similar to that of release of neurotransmitters and to other secretory processes [3, 4].

Chromaffin granules are easily purified from homogenates of adrenal medulla owing to their high density. In the course of an investigation of their light-scattering properties (Morris, S. J., Phillips, J. H. and Edwards, W., unpublished) we observed

Abbreviation: HEPES, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)-ethanesulphonic acid.

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unexpected changes in the ultrastructure of both the cores and the membranes in the presence of the divalent cations Ca^{2+} and Mg^{2+} and these are described in this paper. The observations imply a structural interaction between the core of a granule and its membrane, and the possible relevance of divalent ion-induced changes in this interaction to the secretory process is discussed.

Banks observed that Ca^{2+} causes aggregation of the isolated granules [5], and our light-scattering measurements confirm this. Other divalent cations are also effective, as is lowering the pH. Bovine chromaffin granules aggregate to approximately the same extent (as judged by light scattering) when incubated at room temperature in 0.3 M sucrose at pH 3.5, in 1 mM $CaCl_2$, or in 5 mM $MgCl_2$, conditions which lead to neutralisation of the granules' surface charge [6]. Only in the presence of divalent cations, however, are striking changes in granule morphology found when pellets are examined by electron microscopy.

MATERIALS AND METHODS

Preparation of chromaffin granules

Bovine adrenal glands were cooled on ice and used within 1 h of slaughter. The medullae were minced and homogenised in 0.3 M sucrose containing 10 mM 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)-ethanesulphonic acid (HEPES) buffer, pH 7.0 (this solution is referred to as "buffered sucrose"). Following centrifugation at $1000 \times g$ for 10 min at 4 °C, the homogenate was centrifuged at $27\,000 \times g$ for 20 min at 4 °C. The upper layer of the resulting pellet (mainly mitochondria) was removed [7] and the remainder was resuspended and recentrifuged. The upper layer was again discarded and the resultant pink pellet of crude chromaffin granules was resuspended in buffered sucrose. These granules are fairly stable when diluted in 0.3 M buffered sucrose, and were used for measurements of the release of soluble components.

Chromaffin granules were purified for electron microscopy by centrifugation through continuous sucrose gradients. These contained 1.0–2.3 M buffered sucrose and were centrifuged for 3 h at $200\,000 \times g$ at 4 °C in a swinging bucket rotor (Spinco SW50L). The resultant band of chromaffin granules was collected from the bottom of the centrifuge tube and separated into two halves; only the more dense fraction was used. It was diluted with 0.3 M buffered sucrose to a final concentration of 0.4 M sucrose, and 1 M HEPES was added to a final concentration of 20 mM, pH 7.0.

The purity of these fractions with respect to mitochondrial contamination was assessed by monoamine oxidase assay. 85–90 % of the activity of the supernatant from the low-speed centrifugation is removed by washing of the two subsequent pellets. Monoamine oxidase activity cannot be detected in the purified granules and mitochondria are very rarely observed in electron micrographs of this fraction.

Electron microscopy

Purified chromaffin granules were incubated as indicated in the legends to the figures. Glutaraldehyde was then added to a final concentration of 1 %; the pH of fixation was 7.0. After 15 min at room temperature the granules were collected by centrifugation for 2 h at $200\,000 \times g$ at 4 °C. They were washed with phosphate-buffered sucrose and treated with 2 % OsO_4 before dehydration and embedding in Epon. Sections showing silver or grey interference colours were used. They were double stained with 10 % uranyl acetate in methanol followed by Reynold's lead citrate.

Sections covering the whole depth of the pellet were always examined.

Release of catecholamines and ATP from chromaffin granules

Crude chromaffin granules were lysed by suspension in 10 mM HEPES, pH 7.0. This releases a mixture of protein, catecholamines and ATP which has an absorption maximum at 265 nm. The absorbance after complete lysis is directly proportional to granule concentration. Absorbance at 265 nm was therefore used as a convenient assay for release of the soluble components of chromaffin granules in suspension.

Freshly prepared crude chromaffin granules were diluted into sucrose solutions containing 20 mM HEPES, pH 7.0, at 15 °C. After 5 min each suspension was divided into two: control granules received 7.5 mM NaCl and experimentals received 5 mM $CaCl_2$. After a further 15 min at 15 °C they were taken to ice and immediately centrifuged for 12 min at 27 000 × *g* at 0 °C. Supernatants were removed and their absorbance at 265 nm was determined.

Analytical methods

Protein concentrations were determined as follows: granules were precipitated with ice-cold 5 % (w/v) trichloroacetic acid for 10 min. The resultant pellets were dissolved in 3 % (w/v) NaOH containing 2 % (w/v) sodium deoxycholate [8] and their protein concentration determined by the method of Hartree [9]. Monoamine oxidase was assayed by the method of Wurtman and Axelrod [10] using [^{14}C]tyramine hydrochloride (42 Ci/mole) as substrate.

Materials

Chemicals were obtained from BDH Chemicals Ltd, Poole, Dorset, U.K. [^{14}C]Tyramine hydrochloride was from The Radiochemical Centre, Amersham, Bucks., U.K.

RESULTS

Electron microscopic observations

Control granules, incubated for 45 min at room temperature in 0.4 M sucrose buffered with 20 mM HEPES, pH 7.0 (Na^+ concentration 5 mM), are shown in Fig. 1(a). In general, the granules are intact. Their cores show varying amounts of internal structure: the most electron-dense granules (following glutaraldehyde fixation) presumably contain noradrenalin [11]. However, we have observed in addition that leakage of catecholamines and ATP from damaged granules is accompanied by a loss of electron density and the appearance of an intra-granular substructure, under conditions of protein retention [12, 13]. The majority of granules in Fig. 1(a) have intact membranes which are close to the spherical cores. The membranes appear rather wrinkled, an artefact that arises as a consequence of the purification through high-sucrose concentrations (granules purified through isotonic Ficoll gradients [14] have smooth membranes). Although few intact ghosts are seen in Fig. 1(a), the pellet contains many small membranous vesicles of less than 1000 Å diameter, which may arise from damaged granule membranes.

The addition of Ca^{2+} or Mg^{2+} leads to adhesion of the granules, and the

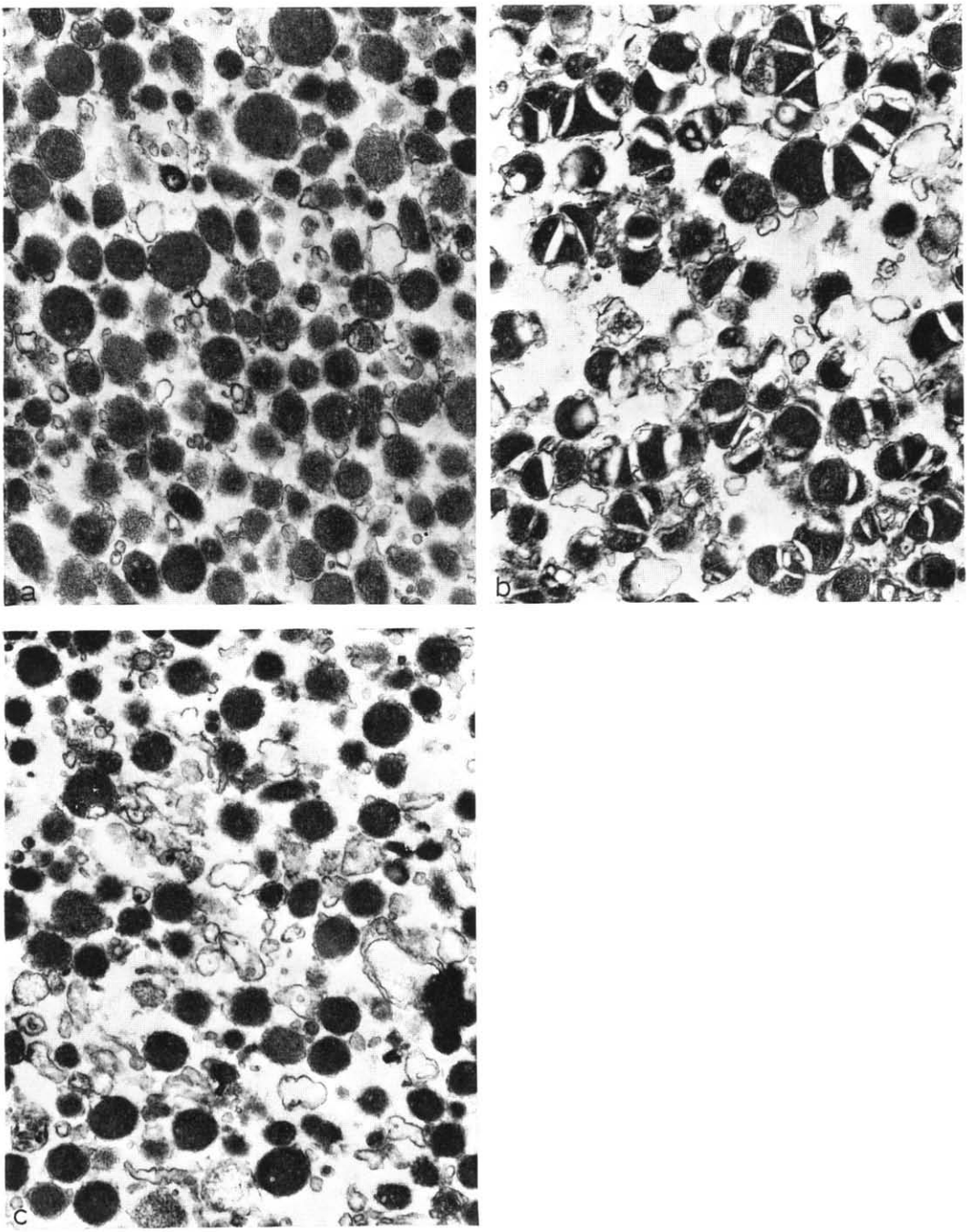

Fig. 1. Reversible aggregation of chromaffin granules by Ca^{2+}. Purified chromaffin granules were suspended in 0.4 M sucrose containing 20 mM HEPES, pH 7.0, at a protein concentration of approximately 75 μg/ml. They were divided into three portions. Control granules (a) received no further additions; one portion (b) received 5 mM $CaCl_2$ (similar results were obtained with 10 mM $MgCl_2$); the third portion (c) received 5 mM $CaCl_2$, followed after 30 min by 10 mM EDTA. After 45 min at room temperature the granules were fixed with glutaraldehyde. Magnification: × 18 000.

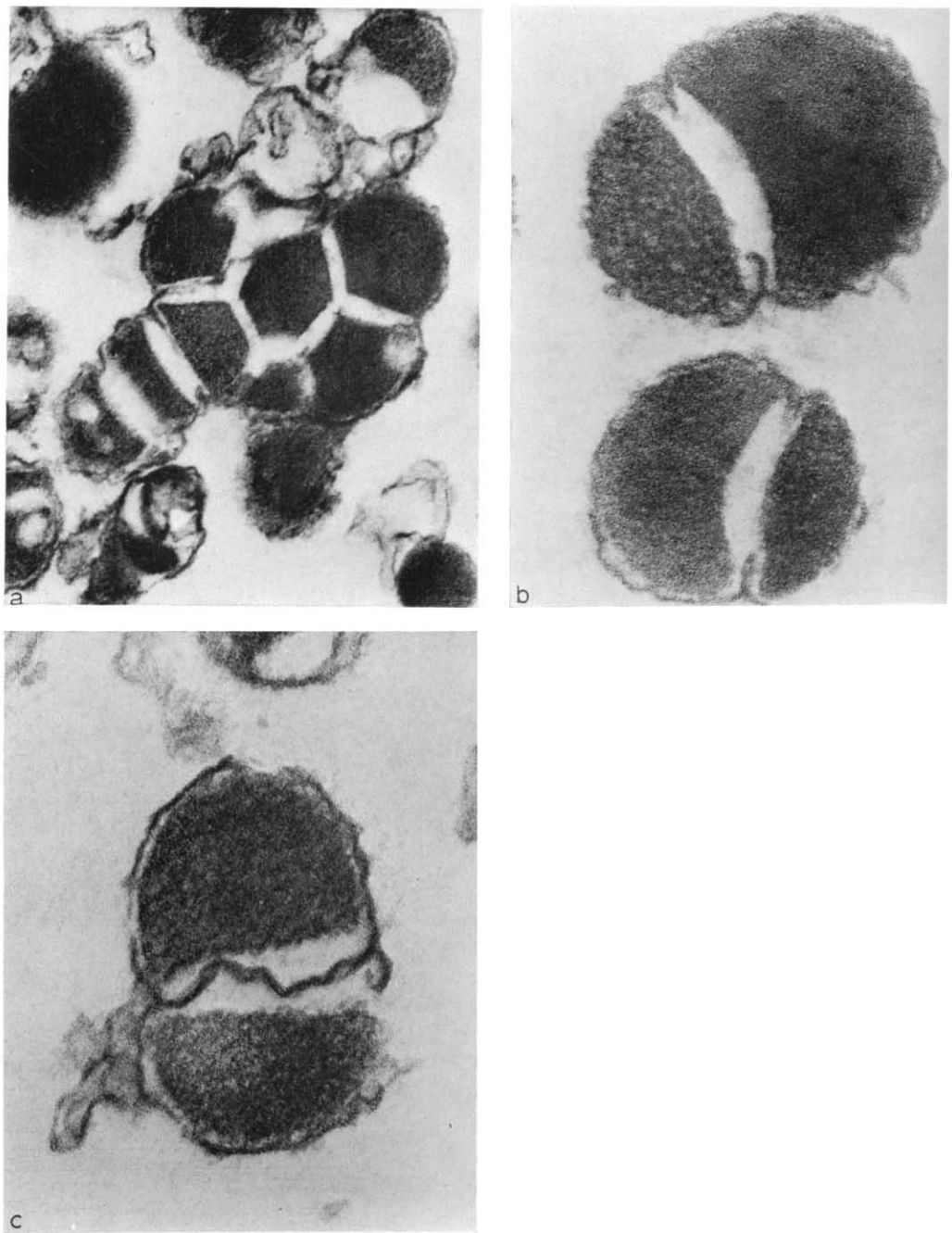

Fig. 2. Chromaffin granule aggregates. Chromaffin granules were incubated for 45 min at room temperature (a) in 0.4 M sucrose containing 20 mM HEPES, pH 7.0, and 5 mM $CaCl_2$ (75 μg protein per ml); or (b and c) in 0.3 M sucrose containing 10 mM HEPES, pH 7.0, 10 mM $MgCl_2$ and 0.1 mM ATP (9 μg protein per ml). This was followed by glutaraldehyde fixation. (a, $\times$ 56 000; b, $\times$110 000; c, $\times$ 113 000).

adhering granules show a reproducible and characteristic change in structure in comparison with control granules (Fig. 1(b)). Their cores are distorted from the usual spherical form, and give the appearance of having retreated from the areas of contact between granules. In sections one sees an electron-lucent "stripe" separating adjacent cores, and the granule membranes, which are close to the cores at areas free from contact with other granules, can often be seen lying within this "stripe" well separated from the cores.

Within the "stripes" one may see two membranes, one membrane or no membrane. In many instances one sees what appear to be broken membranes (Fig. 2). In some instances cores appear to be separated by pentalaminar membranous structures, or the membranes of the granules appear to have become confluent and there is a clear space between the cores, which do not appear to mix with each other. However, in no instance has it been possible to follow trilaminar membrane structures completely through the junction regions.

In some cases in the control field (Fig. 1(a)) granules appear to be extremely close to each other. "Stripe" formation, however, occurs only in the presence of Ca^{2+} or Mg^{2+}.

If there is a sufficient concentration of granules in the suspension, after 45 min at room temperature in 5 mM $CaCl_2$ essentially all the granules are adhering to others. Furthermore, many granules are found in each aggregate; chains are common, but rosettes with a central granule are also found (Fig. 2(a)). The size and extent of these aggregates excludes the possibility that we are seeing the granules dividing into smaller particles.

Suspensions of granules were also treated with 5 mM $CaCl_2$ at 0 °C for 45 min. Examination of these pellets showed that only about 60 % of granules appear in aggregates, and these commonly contain only two or three granules. Long chains and rosettes are not found, presumably for kinetic reasons. Those aggregates that are present, however, resemble those in Fig. 1(b), with deformed cores and "stripes" of similar size: these stripes often contain broken membranes, or appear devoid of membranous material, as seen in Fig. 1 (b). Most of the granules which are not in these aggregates are packed close to each other in the pellet, in some cases with membranes apparently in contact, but without deformation of cores or "stripe" formation.

Fig. 1(c) shows that addition of EDTA after $CaCl_2$ had been added to aggregate the granules at 25 °C leads to disaggregation. The pellet contains many intact granules (note that there were virtually no single granules remaining after $CaCl_2$ treatment, Fig. 1(b)) and more ghosts and debris than in the $CaCl_2$-treated material. Quantitative comparison of this pellet with that of $CaCl_2$-treated granules is difficult, because of the uneven distribution of ghosts through the pellet: the upper layer of the EDTA pellet consists entirely of ghosts, a feature that is virtually absent in controls. Nevertheless, it appears that a substantial proportion of the granules appearing in aggregates after calcium treatment reverts to single intact granules on subsequent treatment width EDTA. This experiment shows (1) that either many granules retain intact membranes: they are not all broken, though all adhere; or, if broken, on withdrawal of Ca^{2+}, many broken membranes reseal without substantial loss of granule contents; (2) that the cores may return to their spherical form with closely fitting membranes; (3) that although some adhering granules remain intact on EDTA treatment, others lyse to yield ghosts and membranous debris; and (4) that

no giant granules or joined granules retaining a "stripe" (like those in Fig. 2(b)) are found after EDTA treatment; this might have been expected if adhering granules as in Fig. 1(b) and 2(b) are, in fact, joined with contiguous lipid bilayers.

Purified 'ghosts' (resealed membranes of granules that have been lysed and separated from core material) aggregate in the presence of $CaCl_2$. Clumps are formed, with membranes in close contact with each other. Granules also aggregate at low pH. After treatment at pH 4 and fixation at this pH, however, no "stripes" were found between adhering granules, unless divalent ions were included in the incubation.

Release of soluble components of chromaffin granules

Catecholamines and ATP leak from chromaffin granules when suspensions are diluted in sucrose solutions. Our electron microscope observations suggested that this leakage should be accelerated in the presence of Ca^{2+} or Mg^{2+}, since granules with broken membranes were commonly seen. Furthermore, this process should be strongly dependent on the concentration of granules if it occurs as a consequence of granule interaction. We tested this by suspending granules in buffered sucrose solutions, in the presence or absence of 5 mM $CaCl_2$. At the same time we investigated the effect of osmotic pressure on Ca^{2+}-evoked release, by using 0.3, 0.4 or 0.5 M sucrose as the suspension medium.

Highly purified chromaffin granules (as used for the electron microscope and light-scattering experiments) cannot be used since they are rather fragile and a high background of lysis makes measurements difficult. The crude granules used, therefore, were somewhat contaminated with other cell organelles. Leakage of catecholamines and ATP was measured by the increase in absorbance at 265 nm of the supernatant after the granules had been removed by centrifugation at 0 °C.

On dilution of the granules to very low protein concentrations it was found that about 10 % of the soluble contents leaked (that is, the absorbance of control samples, Fig. 3, was about 10 % of the absorbance produced by total lysis). This leakage was somewhat less (about 7 %) in the most concentrated suspensions used. Differences between experimentals and controls (that is, Ca^{2+}-evoked release) are shown in Fig. 3. Different batches of granules showed some variation in extent of release and, in general, calcium-evoked release is small compared with the controls. Data from several experiments were therefore pooled.

The results in Fig. 3 show that 5 mM $CaCl_2$ stimulates leakage from the granules above the background value, thus confirming earlier observations of this phenomenon [15, 16]. However, the leakage per granule is greater at higher granule concentrations, suggesting that interaction between the granules is required for Ca^{2+}-evoked release. Finally, the process appears to be highly dependent on osmotic pressure, less release occurring as this is increased. In addition, we have observed that the addition of EDTA following calcium treatment led to an extra release from the granules (results not shown in the figure).

DISCUSSION

The cores of chromaffin granules clearly have some internal structure, perhaps based on a gel of protein molecules; this is shown by the deformed shapes they can assume in aggregating granules (Fig. 1(b)), and by the lack of mixing of the cores

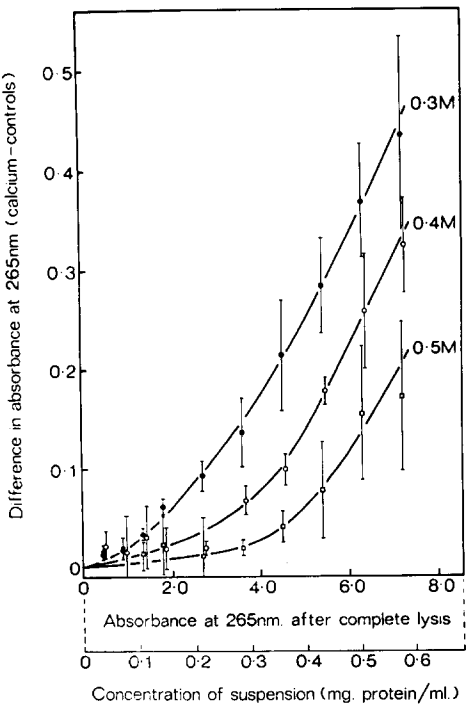

Fig. 3. Dependence of Ca^{2+}-evoked release of catecholamines and ATP from chromaffin granules on granule concentration. Crude chromaffin granules were incubated in the presence of 7.5 mM NaCl (controls) or 5 mM $CaCl_2$ (experimentals) for 15 min at 15 °C. Absorbances at 265 nm of each sample were determined following removal of the granules by centrifugation. Differences between experimentals and controls (i.e. calcium-evoked release) are plotted on the ordinate. The absorbance of supernatants after complete lysis in 10 mM HEPES is directly proportional to granule concentration, and both scales are shown on the abscissa. The points show means (± S.D.) from four experiments (three in the case of 0.5 M sucrose). Sucrose concentrations of the suspension media were 0.3 M (●), 0.4 M (○) and 0.5 M (□). Points from experiments with 0.4 M sucrose have been displaced slightly for clarity.

between joined granules (Fig. 2(b)). The observations also show that there is an adhesion between core and membrane, except in regions of "stripes". This must be quite stable, since small areas of adhesion can maintain the remarkable wedge-shaped cores seen in some places in Fig. 1(b).

Granule aggregation is not a sufficient condition for "stripe" formation, since there are no "stripes" formed at pH 4 unless $CaCl_2$ is present. Our experiments show that Ca^{2+} or, less effectively, Mg^{2+} is required. These act by first promoting aggregation of granules, contact points existing all over the granule surface (Fig. 2(a)). This appears to eliminate interaction between the membrane and the core at this point and triggers dissolution or contraction of the core in this region only, contact being maintained with the rest of the membrane. This gives the appearance of "stripes" and presumably increases the osmotic pressure, inside the granules, in view of the osmotic sensitivity of the Ca^{2+}-evoked release (Fig. 3). Although some adhering granules lyse (Fig. 2(c)), in many cases membranes appear to break at the regions of contact, so that membrane fragments are seen within the "stripes", or, in many cases, the membranes cannot be traced at all (Fig. 2(a) and (b)).

The changes in the core are only found adjacent to the region of inter-granular contact; granules in 5 mM $CaCl_2$ which are not adhering to others do not appear different from controls (as shown both by doing the incubation at 0 °C when not all granules aggregate, or by using more dilute suspensions). The core changes, as well as the aggregation, are reversed by removing Ca^{2+} from the medium with EDTA, when granules that are not too badly damaged revert to their spherical form.

The mere inclusion of divalent ions in the suspension medium is a sufficient condition for producing not only the aggregation of granules but also the changes in core structure and core–membrane interaction and the membrane disruption that we have described. These effects occur in the presence or absence of added ATP, at 0 °C or 25 °C, and at pH 4 or 7.

It is tempting to speculate that the effects of Ca^{2+} and Mg^{2+} which we have observed on isolated granules are equivalent to processes occurring during fusion of granules with the plasma membrane in vivo during exocytosis. Thus a transient influx of Ca^{2+} across the plasma membrane would lead not only to adhesion of granule membranes to the plasma membrane near the point of calcium entry [5], but also to local dissociation of the granule core from its membrane and an increase in the osmotic pressure of the granule, followed by rupture of the adhering membranes.

Although injection of Ca^{2+} intra-axonally promotes neurotransmitter release, Mg^{2+} are not effective in the same way [17]. They do, however, appear to cause adhesion of synaptic vesicles to presynaptic membranes in the frog neuromuscular junction [18], and it is still not clear that plasma membrane release sites have an absolute specificity for Ca^{2+}. Our experiments suggest that, as far as the granule membranes are concerned, non-specific binding sites exist all over the membrane surface. Granule interaction is taken to an extreme stage ("stripe" formation) in vitro and arrested at this point owing to osmotic balance across the adhering membranes, a situation which would not occur during exocytosis. Our conditions also differ from those in vivo in that we have used a high Ca^{2+} concentration in order to facilitate the electron microscopic observations, but light-scattering experiments show that much lower concentrations of Ca^{2+} can lead to granule aggregation. Chromaffin granules do not usually fuse with each other in vivo, presumably because free Ca^{2+} is rapidly sequestered [19], although potential for such fusion does appear to exist with mast cell granules [20] and zymogen granules [21].

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