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PARTICLE SEGREGATION IN CHROMAFFIN GRANULE MEMBRANES BY FORCED PHYSICAL CONTACT

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Summary

Bovine chromaffin granules were exposed to different isotonic non-ionic and ionic solutions (sucrose; Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; Tris-HCl + NaCl; Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline + sucrose; Tris-HCl + sucrose) at pH 7 and then frozen either in suspension or as firm pellets. Freezing was performed without prefixation or antifreeze treatments either by 'standard' techniques (approx. 1 mm^3 suspended or pelleted material on gold specimen supports dipped into liquid Freon) or with increased cooling rates by spraying suspensions into liquid propane ('spray-freezing'). Regardless of the freezing method, membrane-intercalated particles were always randomly distributed when chromaffin granules were frozen in suspension. In contrast, forced physical contact between granules produced by centrifugation (12 000 × *g*, 25 min) provoked dispersal of membrane-intercalated particles, but only in the presence of ions. Sucrose or EDTA in an ionic environment had no inhibitory effect. The following conclusions are derived: (1) Even below the reported phase transition region particle clustering is possible. (2) Chromaffin granule membranes are not liable to thermotropic segregation of membrane-intercalated particles. (3) Although the low freezing rates of 'standard' freezing techniques produce large-scale segregation artefacts (by which suspended chromaffin granules are pushed together within the segregated solute) this does not result in intramembraneous particle segregation. (4) Forced physical contact produces a Ca^{2+}-independent particle segregation, but only when repulsive electrostatic forces of membrane components are partially screened in an ionic environment. (5) This does not invalidate results obtained by others, showing Ca^{2+}-mediated chromaffin granule agglomeration and segregation of membrane-intercalated particles, but it might indicate the occurrence of

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another, not directly Ca^{2+}-dependent particle segregation mechanism in a prefusional stage of close membrane-to-membrane contact during exocytosis.

Introduction

It has been postulated from theoretical considerations that fusion of biomembranes is preceded by a redistribution of membrane-integrated proteins in the fusion zones [1,2]. A method increasingly used to study fusion phenomena is freeze-fracturing. However, it has been shown that this method, when the common 'standard' techniques are used, is liable to produce artifacts of similar appearance. Glutaraldehyde prefixation does not impede, during the subsequent glycerol impregnation, membrane areas being formed which are devoid of membrane-intercalated particles in freeze-fracture analyses [3] or of membrane-integrated proteins in biochemical analyses [4].

The functional importance of particle segregation and the possible formation of artifacts of similar appearance in freeze-fracturing makes it very important to use cryofixation techniques, at least for control experiments, which do not need any preparative pretreatment. In a variety of systems the ultrastructural changes reported to occur immediately before exocytotic membrane fusion are very variable and contradictory, even within the same system, ranging from lack of movement, aggregation or dispersal of membrane-intercalated particles in the presumptive fusion sites (see 'Discussion'). Unfortunately, the applicability of very fast freezing methods is still largely restricted to suspensions of cells or organelles.

In this paper we report on a novel method of inducing lateral particle segregation in membranes of chromaffin granules. Experiments with such isolated secretory vesicles and membranes are becoming increasingly interesting for the analysis of fusion processes occurring during exocytosis. Our observations may be of relevance in this connection, since they could reflect the morphologic situation during a prefusional stage in vivo. We focused our attention on the elimination of artifacts during preparative processing for freeze-fracturing.

Materials and Methods

Partly purified chromaffin granules were isolated from bovine adrenal medullae by differential centrifugation [5]. A large granule fraction was sedimented with 12 000 $\times g_{max}$ for 25 min. The brownish top layer was removed and the sediment resuspended. This procedure was repeated once. The remaining partly purified chromaffin granules were resuspended in different media (0.3 M sucrose; Dulbecco's Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, pH 7.0; Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline + 0.3 M sucrose; Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline + 10 mM EDTA; 10 mM Tris HCl, pH 7.0, + 0.3 M sucrose; 10 mM Tris HCl + 0.9% NaCl) and incubated for 30 min at 2 or 20°C. The same procedures were applied for pilot experiments with membranes isolated from chromaffin granules. To obtain these, highly purified chromaffin granules were isolated by centrifugation through 1.8 M sucrose [5,6]. Membranes of these granules were isolated according to ref. 7 after

hypotonic lysis in Tris/sodium succinate buffer (0.005 M, pH 5.9) and freezing and thawing in three repeated washing cycles.

For freeze-fracture experiments no pretreatment with fixatives or cryoprotectants was applied. After centrifugation (12 000 × g_{max} 25 min) the excess of fluid was removed and approx. 1 mm^3 samples of the pellet were transferred with a glass rod without stirring onto temperature-adjusted golden specimen supports (Balzers type). Aliquots were processed in suspension by pipetting approx. 1 mm^3 samples onto gold holders or by 'spray freezing' from a preselected temperature. Some samples were repeatedly centrifuged and resuspended, both at 2°C and at 20°C (the temperature was of no importance for the results obtained), in the same solution before freezing by any one of the methods indicated. Specimens mounted on golden holders were quickly pushed into melting Freon 22 of −160°C ('standard' freezing), while liquid propane (−190°C) served as a coolant (for merely practical reasons) for 'spray freezing'. This was performed with a non-commercial unit as indicated by Bachman and coworkers [8,9,10], using *n*-butylbenzene as adhesive (−85°C). 'Standard' freezing of 1 mm^3 samples represents a much slower freezing mode than 'spray-freezing' [8].

All samples were processed with a Balzers unit, type BAF 300, equipped with a turbomolecular pump and electron beam evaporators for platinum-carbon and carbon evaporation, which was carried out immediately after cutting at 10^{-6} Torr and −100°C. In Figs. 1–6 the shadowing direction is indicated by an arrowhead in the lower left corner.

For controls some samples were processed by routine electron microscopic fixation (glutaraldehyde, OsO_4), embedding (epoxide resin), ultrathin sectioning and staining (uranyl acetate and alkaline lead citrate) techniques.

Results

The results obtained are summarized in Table I and some characteristic situations are illustrated in Figs. 1–6. In all cases membrane-intercalated particles were much more abundant on the concave (outer) fracture halves of chromaffin granule membranes than on the other side (see also refs. 11 and 12).

In the absence of an ionic medium, i.e., in sucrose alone, membrane-intercalated particles were never aggregated whether chromaffin granules were frozen loosely suspended or as a firm pellet. When frozen in suspension, particles were always randomly distributed (Figs. 1 and 2) also in an ionic medium. The temperature at which centrifugation or incubation with the different solutions was carried out, as well as the type of freezing procedure used, remained without any detectable effect on the arrangement of membrane-intercalated particles. Compare Fig. 1 with Fig. 2 for spray-freezing versus standard freezing.

Experiments in which chromaffin granules were incubated in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline or Tris-HCl buffer gave the following results. The mode of particle distribution observed is independent of the type of buffer, starting temperature and freezing rate. Therefore, the sole parameter which determines whether particles are randomly distributed or clustered in the chromaffin granule membranes is the state of dispersal of chromaffin granules,

TABLE I

INFLUENCE OF INCUBATION MEDIA, STARTING TEMPERATURE, PACKING DENSITY AND MODE OF CRYOFIXATION ON THE DISTRIBUTION PATTERN OF MEMBRANE-INTERCALATED PARTICLES IN BOVINE CHROMAFFIN GRANULES

CMF-PBS, Ca^{2+} and Mg^{2+}-free phosphate-buffered saline.

Treatment of chromaffin granules	Mode of cryofixation	Distribution pattern of membrane-intercalated particles
(A) Non-ionic media		
Sucrose: 20°C, firm pellet	gold holders (Freon 22)	random
Sucrose: 2°C, firm pellet	gold holders (Freon 22)	random
Sucrose: 20°C, suspension	spray-freezing (propane)	random
Sucrose: 2°C, suspension	spray-freezing (propane)	random
(B) Ionic media		
CMF-PBS: 20°C, firm pellet	gold holders (Freon 22)	segregated
CMF-PBS: 2°C, firm pellet	gold holders (Freon 22)	segregated
CMF-PBS: 20°C, suspension	gold holders (Freon 22)	random
CMF-PBS: 2°C, suspension	gold holders (Freon 22)	random
CMF-PBS: 2°C, suspension	spray-freezing (propane)	random
CMF-PBS: 2°C, suspension → pellet	gold holders (Freon 22)	segregated
CMF-PBS: 2°C, suspension → pellet → suspension	spray-freezing (propane)	random
CMF-PBS + sucrose: 20°C, firm pellet	gold holders (Freon 22)	segregated
CMF-PBS + sucrose: 2°C, firm pellet	gold holders (Freon 22)	segregated
CMF-PBS + sucrose: 20°C, suspension	gold holders (Freon 22)	random
CMF-PBS + sucrose: 2°C, suspension	gold holders (Freon 22)	random
CMF-PBS + sucrose: 2°C, suspension	spray-freezing (propane)	random
Tris-HCl + 0.9% NaCl: 2°C, firm pellet	gold holders (Freon 22)	segregated
Tris-HCl + 0.9% NaCl: 2°C, suspension	gold holders (Freon 22)	random
Tris-HCl + sucrose: 20°C, firm pellet	gold holders (Freon 22)	segregated
Tris-HCl + sucrose: 2°C, firm pellet	gold holders (Freon 22)	segregated
Tris-HCl + sucrose: 20°C, suspension	gold holders (Freon 22)	random
Tris-HCl + sucrose: 2°C, suspension	gold holders (Freon 22)	random
CMF-PBS + 10 mM EDTA: 2°C, firm pellet	gold holders (Freon 22)	segregated
CMF-PBS + 10 mM EDTA: 2°C, suspension	gold holders (Freon 22)	random

provided there is an ionic medium. In any ionic medium membrane-intercalated particles are clustered only in firm pellets, not in suspensions.

After centrifugation chromaffin granules are slightly compressed, as one can frequently recognize from the occurrence of flat contact areas delineated by 'edges' (Fig. 3). Membrane-intercalated particles are segregated predominantly onto the edges, but also frequently onto the central region of flat contact areas, where they might form rosette-like clusters (Figs. 4, 5). Sometimes, membrane-intercalated particles, present on outer membrane fracture halves, were in register with corresponding, relatively large pits on inner membrane fracture halves (Figs. 4 and 6). It looks as if particles were 'squeezed out' as far as possible from the zones of most intimate contact between two adjacent chromaffin granules, be it onto the edges or onto restricted areas of membrane-to-membrane apposition, where membranes might be slightly separated and where rosette-like structures could be formed. On ultrathin sections the contact zones presented themselves as pentalaminar structures (with interruptions), resembling the appearance of the prefusional stage of intimate membrane apposition occurring before exocytotic membrane fusion in a variety of systems [13].

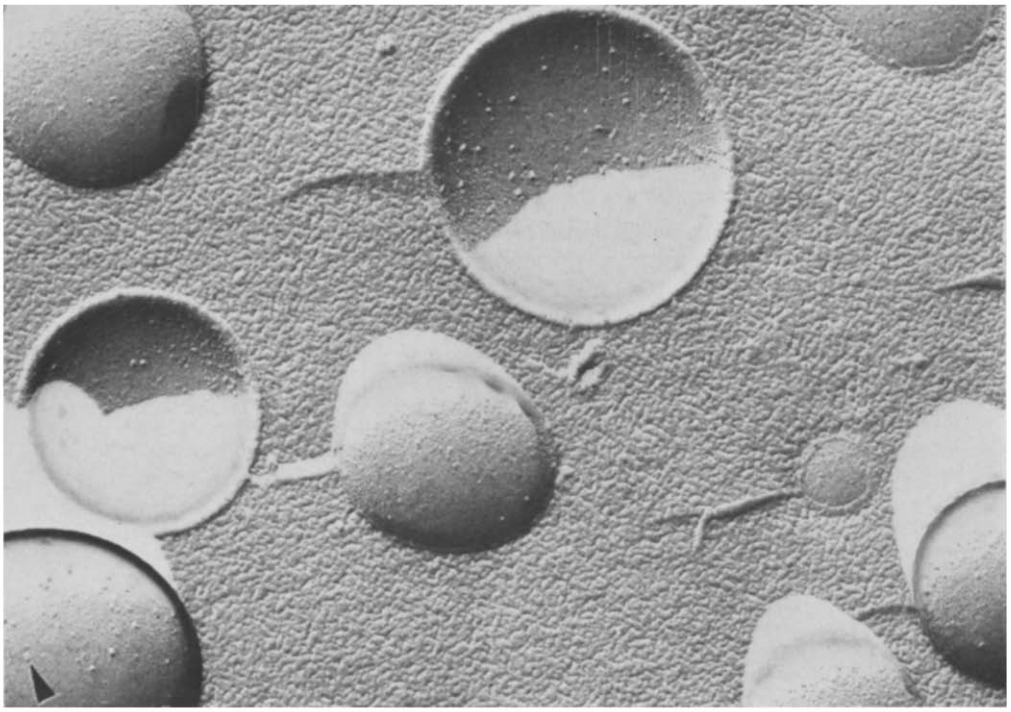


Fig. 1. Isolated chromaffin granules suspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and spray frozen. The background granularity is only 20—30 nm. Chromaffin granule membranes contain more membrane-intercalated particles on their concave (outer) fracture half than on the convex (inner) one. Particles are randomly distributed. Magnification, approx. 55 000X.

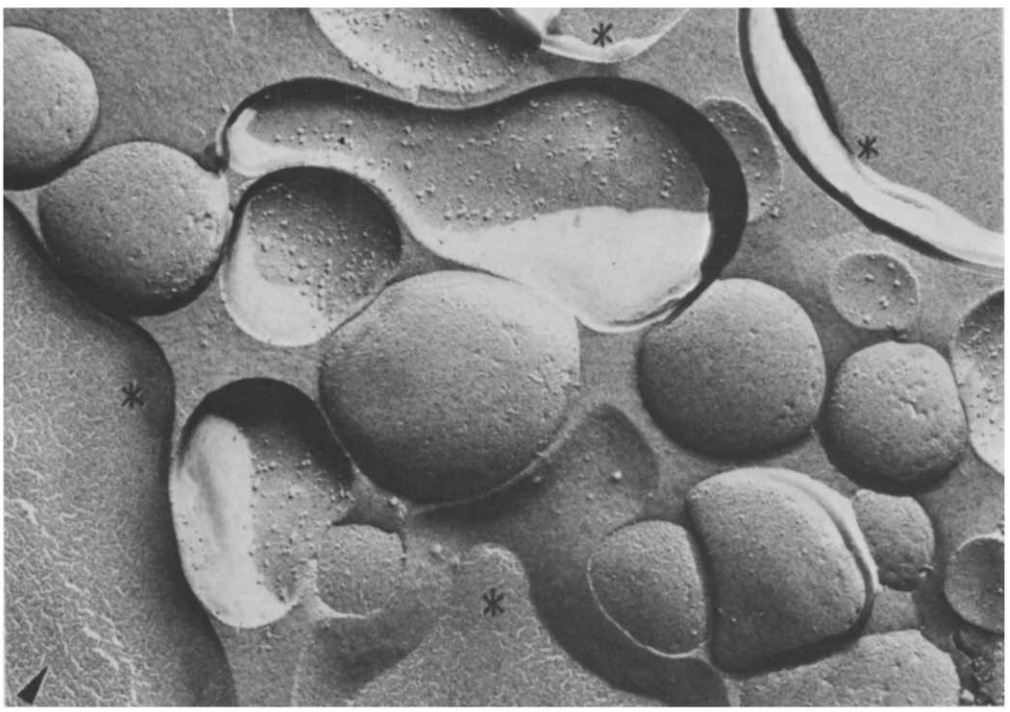


Fig. 2. Chromaffin granules in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline frozen in suspension on gold holders in Freon 22. In the course of large-scale phase separation (due to inferior cooling rates in the absence of antifreeze agents) ice-crystals, labeled by asterisks, have formed and surround the segregated solute phase. Within the latter, suspended chromaffin granules were closely pushed together. Particles remained randomly distributed on both membrane fracture halves despite the large scale phase separation,

Fig. 3. Chromaffin granules pelleted in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline and frozen on gold holders in Freon 22. Chromaffin granules are frequently apposed to each other. Zones of intimate membrane-to-membrane contact, which are especially easy to recognize, where membrane remnants of a chromaffin granule remained attached to the membrane of an underlying granule (asterisks), are devoid of membrane-intercalated particles. These are clustered in regions outside the close membrane-to-membrane contact zones. Magnification, approx. 76 000X.

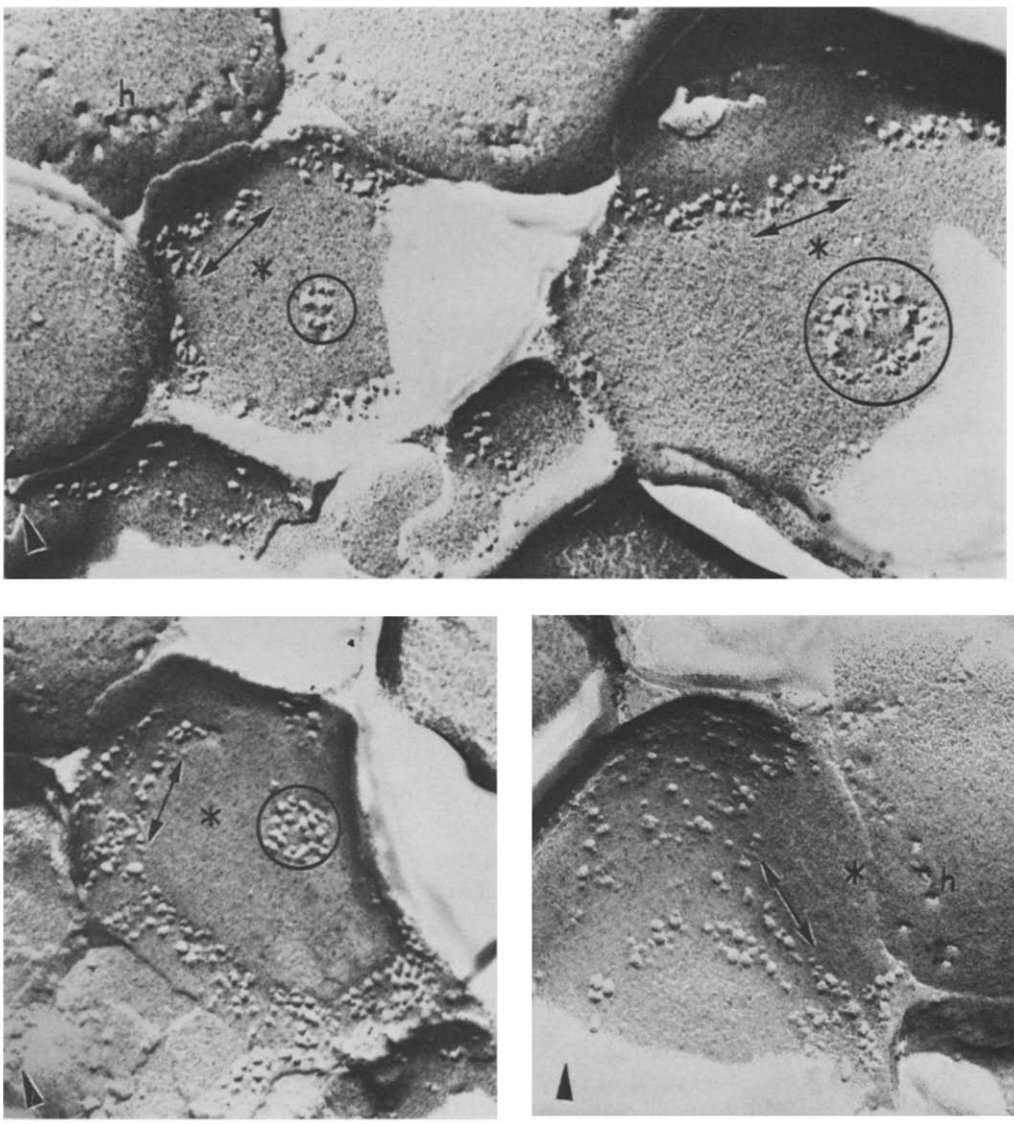
Experiments with sucrose added to buffer solutions prove that sucrose does not exert any inhibitory effect on the clustering of membrane-intercalated particles (Table I).

After standard freezing no particle segregation was observed with chromaffin granules frozen in suspension, even when granules themselves were mostly considerably segregated and clumped within the segregated solute phase due to ice-crystal growth in the surrounding aqueous medium with moderate freezing rates (Fig. 2). The clumping of chromaffin granules was avoided and ice-crystal growth and segregation in the medium was restricted to a small background granularity of about 20—30 nm, when the same granule suspensions were processed by spray freezing (Fig. 1), i.e. with increased cooling rate. Segregation of membrane-intercalated particles did not occur with any of the freezing methods (freezing rates) used in the present study.

Routine biochemical controls render it very unlikely that the unequivocal and persistent particle clustering, observed in the majority of chromaffin granules after freezing as a firm pellet in an ionic medium, would be due to membrane ruptures. The following experiments further rule out this theoretical possibility. Particle clustering is reversible when pelleted granules are resuspended, even at 2°C. Experiments with Ca^{2+}- and Mg^{2+}-free phosphate-buffered

saline plus 10 mM EDTA added disprove the possibility that particle clustering would be due to calcium-leakage from the chromaffin granule matrix. When granules were lysed by the procedure indicated under Materials and Methods, i.e. by repeated freezing and thawing in a hypotonic buffer, membrane-intercalated particles were randomly distributed after spray freezing in suspension.

Particle clustering was such a consistent phenomenon, occurring with practi-



Figs. 4—6. Same preparation as in Fig. 3, showing additional structural details occurring after centrifugation. Membrane-intercalated particles sometimes form patchy clusters (encircled areas) which correspond probably to zones of less intimate membrane-to-membrane contact within an extended contact zone (asterisks). Particles were 'squeezed out' onto the 'edges' (arrows) of contact areas between adjacent chromaffin granules. h denotes holes on the inner membrane fracture half; the holes correspond to membrane-intercalated particles on the complementary outer membrane fracture half. Magnification, approx. 135 000X.

cally all chromaffin granules (at regions where intimate contact was recognizable between adjacent granule membranes), that no statistical approach was necessary to ascertain this phenomenon.

Discussion

Membrane-intercalated particles represent membrane-integrated proteins (c.f. ref. 14) suspended in the two-dimensional liquid lipid bilayer [15]. In biomembranes lipids undergo a phase transition from the liquid to the solid gel state at certain temperature regions [16]. In most biomembranes particles are 'frozen out' along phase boundaries formed upon transition [17]. In negatively charged lipids Ca^{2+} also infers a higher degree of order and increases transition temperatures [18]. Because of the presence of negative surface charges in chromaffin granules (see below) and the importance of Ca^{2+} for exocytosis [19,20] phase separation phenomena deserve some attention in membrane fusion phenomena.

Although the phase transition region is rather high, namely around 32—36°C, in bovine chromaffin granules [21,22], membrane-intercalated particles evidently still retain considerable translational mobility at low temperatures, since they can be clustered even at +2°C (Table I; see also ref. 23). Alternatively we could not induce particle clustering by thermotropic phase transition.

Chromaffin granules discharge their contents by exocytosis (c.f. ref. 24), i.e. by temporary fusion of the granule membrane with the cell membrane. It appears feasable to investigate membrane interactions between isolated chromaffin granules and to assume that similar ultrastructural changes might occur in these membranes during exocytosis. Exocytosis frequently involves also granule-to-granule interactions, which then discharge as a continuum ('compound exocytosis', [19]); furthermore, the pentalaminar membrane contact structure seen on ultrathin sections of chromaffin granules in forced physical contact is similar to that seen before exocytosis in a variety of systems [13].

Exocytosis involves 'stimulus-secretion-coupling', i.e. a Ca^{2+}-influx upon stimulation [19,20]. There is no consense on the role(s) of Ca^{2+} during exocytosis. Ca^{2+} could screen the negative surface charges of chromaffin granules [25] and, as far as present there, equally at the inner side of the cell membrane. This could allow for both membranes to come into close contact with the formation of ionic linkages between both membranes. In vitro, Ca^{2+} agglomerates isolated bovine chromaffin granules and membrane-intercalated particles are segregated from the sites of membrane contact [23]. This could be explained theoretically by a Ca^{2+}-induced solidification of membrane lipids [18] during which particles would be 'frozen out' from the Ca^{2+}-binding membrane contact sites. This is opposite to the widely held view that membranes would have to be in a fluid state, or even selectively perturbed [1,2], at fusion sites. It also contrasts with the occurrence of particle clustering in the absence of Ca^{2+} (this paper).

In the present case, the segregation of membrane-intercalated particles was independent of temperature and calcium. Our findings, i.e the clustering of membrane-intercalated particles by forced physical contact in an ionic medium, could then be tentatively interpreted in the following sense: normally the particles would be kept in a random distribution by electrostatic repulsive forces.

Only when chromaffin granules are pressed into intimate contact with each other and when an ionic medium provides a partial charge screening, the membrane-intercalated particles can be squeezed together outside the membrane contact zones. Since this effect takes place even in the presence of EDTA, it cannot be accounted for by Ca^{2+} (even if some Ca^{2+} would leak out from the matrix). It could be speculated that the local elimination of membrane-intercalated particles from the membrane contact zones reflects a situation, which might also occur before membrane fusion in vivo. Indeed, a prefusional dispersal of membrane-intercalated particles has been postulated for membrane fusion to occur [1,2]. If this holds true, Ca^{2+} could interfere in vivo on another level, e.g. by regulating contractile forces via cytoskeletal elements, and our experiments would have mimicked this effect in vitro. Further investigations along these lines will be needed to substantiate this speculation.

The number of membrane-intercalated particles in bovine chromaffin granules is rather small and they are mostly asymmetrically distributed in such a way that they do not penetrate the whole membrane. It appears, therefore, difficult to correlate any ultrastructural details with the extremely high lysophosphatidylcholine content of these secretory granules [26].

We alluded in the Introduction to the problem of particle segregation not only being potentially an important feature of membrane fusion but possibly occurring also as a preparative artifact. The usual method of cryofixation, i.e. dipping an approx. 1 mm^3 sample, mounted on a gold specimen support, into an organic coolant, does not freeze biological specimens fast enough to avoid large-scale segregation phenomena [14]. Its most disturbing form, ice-crystal damage, is reduced in standard preparation techniques by impregnation with an antifreeze agent. It was cautioned very early that antifreeze treatment in conjunction with too low cooling rates can introduce less obvious intramembranous segregation artifacts [27], even in highly viable cells. Therefore it has become common practice to stabilize biological materials by glutaraldehyde fixation to render them resistant to the subsequent antifreeze treatment. Alternatively, the spray freezing method [8] allowed to increase the cooling rate for suspended cells and subcellular fractions [9,10], so that there was no longer any need for any petreatment; intramembranous segregation artifacts of membrane-intercalated particles were also abolished by this mode of cryofixation [10,27].

The continuous use of standard cryofixation techniques in other laboratories also resulted in the observation of particle segregation upon antifreeze treatment [28] and it was invoked that glutaraldehyde prefixation abolished this artifact. It then became a widely held assumption that aldehyde prefixation and glycerol impregnation allows for the visualization of membrane ultrastructure by freeze-fracturing with a sufficient degree of reliability. In recent reports (see e.g. ref. 3) it was again cautioned that aldehyde prefixation cannot always sufficiently stabilize the membrane structure to avoid the segregation of membrane-intercalated particles during subsequent glycerol impregnation.

In an early study we freeze-fractured cat adrenal medullae (glycerol treated but unfixed) and isolated bovine chromaffin granules (without pretreatment), [12]. With granules in sucrose the morphology was identical with that presented here. Although we observed in situ a close apposition of peripheral granules to the cell membrane, neither membrane displayed any obvious speciali-

zation at the contact sites. Reinvestigations (using glutaraldehyde fixation and glycerination) of hamster adrenal medullae with a high amount of spontaneous exocytotic profiles eventually, after stimulation, also failed to visualize local membrane changes [29]. With other endocrine cells the prefusional stage was characterized as involving an aggregation of membrane-intercalated particles over presumable fusion sites [30—32], but the opposite, i.e., the clearing of membrane-intercalated particles from presumptive fusion zones, was also repeatedly reported (see, for instance ref. 33). Undoubtedly it is difficult in most systems to define clearly enough the functional stage of a secretory granule even if it is closely apposed to the plasmalemma. In view of the tentative interpretation given above our results are in favour of a prefusional dispersal of membrane-intercalated particles, at least in the secretory granule membrane.

Recently Wattiaux-DeConinck et al. [34] presented evidence for the segregation of membrane-intercalated particles in mitochondria (contained in a sucrose-imidazole solution) at low temperature due to a pressure-induced increase of the phase transition temperature. We consider this an effect different from that observed with chromaffin granules because of the following reasons: (a) the pressure (per granule contact area) generated here can be estimated to be several orders of magnitude below that in ref. 34; (b) in our system particle clustering persisted, even when samples were de-pressurized for 30 min (unpublished observations); (c) no particle clustering occurred in chromaffin granules in sucrose, even when pressurized; (d) with chromaffin granules the effect was independent from the starting temperature and thermotropic effects do not seem to play any role in particle segregation in chromaffin granule membranes.

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