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Hyperosmotic relaxation lysis of chromaffin granules is caused by interactions between the granular membrane and intragranular vesicles

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Bovine chromaffin granules undergo irreversible structural changes during osmotic shrinkage in hypertonic sucrose and salt solutions, such that, on reexposure to isoosmotic conditions they do not regain their original morphology, but undergo lysis ('hyperosmotic relaxation lysis'). Irreversible alterations of granules were induced by hypertonic incubations lasting for as little as 1 min. Fluorescence and EPR membrane labelling experiments showed that hypertonicity did not induce membrane loss for instance by inwardly or outwardly directed pinching off of membrane material. The mean sizes of chromaffin granules as a function of increasing and subsequently decreasing osmotic pressure were measured by photon correlation spectroscopy; there was no significant difference in sizes of hyperosmotically pretreated granules as compared with controls. Freeze-fracture electron micrographs showed the formation of 'twins' and 'triplets' under hypertonic conditions. They also revealed intragranular vesicles of 50–200 nm in diameter in both hypertonically and isotonically suspended granules. 'Twin' and 'triplet' granules were formed by the attachment of intragranular vesicles to the granule membranes. We suggest that hyperosmotic relaxation lysis is caused by the fact that this adhesion partly prevents the granule membrane from reexpanding, thus, leading to its rupture.

Introduction

Chromaffin granules are the storage vesicles in adrenal medullary cells. They have been frequently used as model systems for the investigation of hormone accumulation, storage and exocytosis. Osmotic properties of chromaffin granules have been intensively investigated [1–4] since osmotic forces play an important role in exocytosis [5–16]. However, the mechanism by which osmotic pressure gradients facilitate exocytosis is not known [16]. It has been suggested that osmotic forces either cause membrane fusion and fission directly, by stretching the secretory vesicle membrane [6,7,17–19], or are of importance in facilitating release of the contents after fusion [11,12].

Isolated chromaffin granules are stable in isotonic (0.3 osM) sucrose solutions. In hypotonic solutions the granules undergo hypoosmotic lysis with half-maximal catecholamine release at approx. 200 mosM. In hypertonic sucrose solutions the granules remain intact for hours at low temperatures and do not release their contents [20]. They shrink and behave as almost ideal osmometers at least with respect to increasing osmotic pressure [1,3]. However, if they are transferred back to lower but still hypertonic osmolarities, at which they were previously stable, lysis and hormone release occurs [1,4,20]. In the past, this phenomenon was frequently observed when isolated chromaffin granules were purified by sucrose density gradient centrifugation and resuspended in isotonic solutions (see Ref. 21). Südhof [1] called it 'hyperosmotic relaxation lysis'. It does not appear to be caused by sucrose uptake into the granules [1], neither it is specific for sucrose solutions. It was also observed in hypertonic NaCl solutions [1]. There is as

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; EDTA, ethylenediaminetetraacetic acid; osM, osmolar; IMP, intramembranous particle; ONS, 2-*n*-octadecylamino-naphthalene-6-sulphonic acid; TNBS, 2,4,6-trinitrobenzenesulphonate; CAT 16, 1-oxyl-2,2,6,6-tetramethyl-4-dimethylaminopiperidinecetyl bromide; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

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yet no satisfactory explanation for hyperosmotic relaxation lysis.

Because of the apparent importance of osmotic forces in exocytosis we reinvestigated this unusual phenomenon of hyperosmotic relaxation lysis. We tested different hypotheses regarding the structural changes of granules caused by hypertonic incubation, using the following methods: fluorescence catecholamine release assay, fluorescence- and EPR-labelling techniques, photon correlation spectroscopy, and freeze-fracture electron microscopy.

We used fluorescence-labelling techniques and EPR measurements to determine whether there was a loss of membrane material due to endocytotic pinching-off during hyperosmotic incubation. No decrease in granule membrane area was detected. The mean sizes of granules as a function of osmotic pressure were measured by photon correlation spectroscopy; no significant difference in the sizes of granules that either were or were not preincubated at still higher osmolarities was observed at the same osmotic pressure. We also made freeze-fracture electron micrographs, which revealed that 'twin' and 'triplet' structures were formed out of originally spherical vesicles in hypertonic media. These shapes were probably caused by adhesion of small intragranular vesicles [22] to the granule membranes. Hence, when these membranes came into close contact and adhered strongly then the outer granule membrane was only partly available for osmotic swelling. After a decrease of osmotic pressure the granules were therefore unable to round up again, and swelling back to their original size and shape was prevented.

Materials and Methods

Solutions. All solutions were buffered with 10 mM Na-Hepes, Serva, at pH 7.4. The osmotic pressure of buffered isotonic (0.26 M) sucrose solution was 0.299 osM measured by a Knauer osmometer. All other osmolarities as well as refractive indices and viscosities of sucrose solutions were taken from Ref. 23, (p. D-262, Table 88).

Preparation of chromaffin granules. Granules were prepared as described by Südhof [1]. This procedure yielded a crude (P_2) granule fraction, but contamination with other organelles was comparatively low as shown by electron microscopy. Bovine adrenal glands were obtained from a local slaughterhouse and placed on ice within 30 min after death of the animal. Preparation started 1 h later. After dissection and homogenization of adrenal medullary tissue in isotonic buffered sucrose solution, cell debris was spun down at $1000 \times g$ for 10 min. The granule-containing supernatant was centrifuged at $22000 \times g$ for 20 min and the pellet (P_2) was carefully resuspended in isotonic sucrose, whilst leaving behind a thin brown layer (mainly mitochondria). This procedure was repeated twice. The final pellet was resuspended in a small volume of sucrose solution and centrifuged at $2000 \times g$ for 5 min. The resulting homogeneous supernatant contained about 5–10 mg protein/ml as determined by the biuret method with human serum albumin as standard and was used for the following experiments. Isolation was carried out at 4 °C.

Lysis determination. Lysis of chromaffin granules was determined by a fluorimetric catecholamine release assay [1,24]. Catecholamines show maximum fluorescence excitation at 285 nm and emission at 320 nm. As catecholamines are very concentrated within the granules (0.5–0.7 M) [25,26] their fluorescence is quenched. Thus dilution of catecholamines due to lysis is accompanied by an increase of fluorescence. 0% lysis refers to the fluorescence intensity of an aliquot of granules in isotonic or hypertonic solutions whereas 100% lysis refers to the intensity in 10 mM Hepes.

Measurements were carried out in a Perkin-Elmer MPF 4B fluorescence spectrometer using wavelengths of 285 nm (excitation) and 320 nm (emission) and slit widths of 6 nm. Granules were added, either from the stock suspension or after hypertonic incubation, into 2 ml of sucrose solution in a quartz cuvette at 16 °C. The final protein concentration was less than 200 μg/ml. After 2 min equilibration spectra were read and corrected for the corresponding sucrose solution.

Membrane labelling by fluorescent and spin probes. For fluorescence measurements [27] the granule membranes were labelled with 0.2 mM ONS (2-*n*-octadecylaminonaphthalene-6-sulphonic acid), Serva, for 15 min. ONS was dissolved in a 1 : 1 mixture of dimethylsulfoxid and ethanol. After centrifugation at $20000 \times g$ for 20 min the granules were incubated in isotonic or hypertonic sucrose solutions. After 15 min 1.4 mM freshly dissolved TNBS (2,4,6-trinitrobenzenesulphonate) was added, and reacted with the granules for 1 h at 4 °C [27,28]. After centrifugation at $20000 \times g$ for 20 min the granules were resuspended in the appropriate sucrose solution and ONS fluorescence was read using wavelengths of 368 nm (excitation) and 425 nm (emission).

In EPR experiments, granules were first gently mixed with 1 mM of the cationic spin probe CAT 16 (1-oxyl-2,2,6,6-tetramethyl-4-dimethylaminopiperidinecetyl bromide), Bulgarian Academy of Sciences, Sofia, at 4 °C for 30 min [29]. The molar ratio label/lipid was about 1 : 50. The lipid content of the granules was determined by the ammoniumferrothiocyanate method [30]. After centrifugation at $16000 \times g$ for 10 min granules were resuspended in ice-cold solutions of different sucrose concentrations. Immediately before the recording of spectra ascorbic acid was added to the samples at a final concentration of 1 mM. The temperature of the cuvette was adjusted to 4 ± 0.5 °C. In order to determine the redox activity of the granules, the signal

stability of the water-soluble spin probe TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) was measured in parallel experiments. Spectra were read at an ZWG 231 EPR spectrometer (Academy of Sciences of the G.D.R.) at 3290 G, a modulation of 8 G, and a phase of 2/10–2/20.

Both fluorescence and EPR labelling experiments were carried out with two different granule preparations.

Photon correlation spectroscopy. Measurements of $1/z$ average diameters of granules were performed using a 'Zetasizer-2' (Malvern, U.K.) with the 632.8 nm line of a helium-neon laser. Granules were suspended in buffered sucrose solutions, containing 0.5 mM EDTA, at a concentration (50 μg protein/ml) that should be low enough to avoid secondary scattering or granule aggregation [31]. Buffer and sucrose solutions were prefiltered through 0.22 μm filters, Millipore Corp. Granules were incubated and diluted with 4°C solutions whereas measurements were carried out at 20°C. Scattered light signals were accumulated over a period of 10 min for each sample. For data analysis the method of cumulant expansion [32] was used, which yielded $1/z$ average diameter as well as polydispersity.

Freeze-fracture electron microscopy. Granules were prepared as described above and then incubated at different osmotic pressures for 15 min. Some samples were fixed with 2% glutaraldehyde at 20°C for 20 min; usually the samples were used without fixation. Granules were centrifuged for 20 min at 22000 × g and kept at 0°C. A sandwich technique was used, in which the material was placed between specimen holder plates for the double-replica stage of Balzers. After freezing by plunging into liquid propane the samples were fractured and replicated at −150°C using a BAF 400 D apparatus (Balzers AG, Liechtenstein). The replicas were cleaned with sodium hypochlorite and rinsed with distilled water. They were examined in a JEM 100 B electron microscope (Jeol, Japan) at 80 kV.

Results

Lysis experiments

Repeating several experiments of Südhof [1] we were especially interested in the dependence of hyperosmotic relaxation lysis on the incubation osmolarity. Fig. 1 shows the lysis of chromaffin granules, in terms of catecholamine release, as a function of external sucrose concentration after transfering the granules from hypertonic solutions to the lower osmolarities indicated. Granules were preincubated in solutions of different sucrose concentration for 15 min, and no catecholamine release was observed when the granule suspension was diluted with the incubation solution (see 0% values for incubation concentration). Lowering the sucrose concentration, however, led to hyperosmotic relaxation lysis. The leftmost curve of Fig. 1 refers to hypoosmotic lysis, diluting the granule suspension from isotonic sucrose concentration to lower osmolarities. There is a monotonic change of the slopes of the lysis curves, in that the curves become steeper the lower the concentration of sucrose in the incubation.

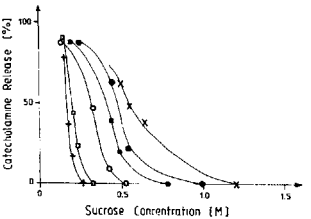

Fig. 1. Hyperosmotic relaxation lysis of chromaffin granules in terms of catecholamine release as a function of sucrose concentration. Granules were preincubated in 0.26 (+), 0.39 (□), 0.52 (○), 0.78 (■), 0.99 (●) or 1.2 (×) M buffered sucrose at 4°C for 15 min. A transfer to the respective solution of lower sucrose concentration followed. The leftmost curve starting from isotonic solution (0.26 M sucrose) refers to hypotonic lysis; the other curves reflect hyperosmotic relaxation lysis.

It should be noted that maximum catecholamine release due to hyperosmotic relaxation lysis never reached the value of hypoosmotically lysed granules (at 0 M sucrose concentration). Only 75–90% catecholamine release as compared with hypoosmotic lysis was observed (see also Ref. 1).

We tested whether hyperosmotic relaxation lysis was caused by the 'dilution shock' occurring suddenly when transfer from high to low osmotic pressure was accomplished. Granules were preincubated at 0.78 M sucrose

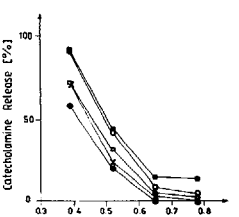

Fig. 2. Influence of the incubation time in 0.78 M sucrose on hyperosmotic relaxation lysis of chromaffin granules. Granules were transferred from the incubation solution to the respective solution of lower sucrose concentration in one step after 1 min (●), 3 min (×), 15 min (□), 60 min (○) or 150 min (■).

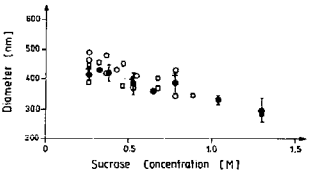

Fig. 3. $1/z$ average diameter of chromaffin granules as determined by photon correlation spectroscopy. ●, mean ± S.D. of control granules from four different preparations suspended at the indicated sucrose concentration. The other symbols refer to granules of two different preparations preincubated at 0.78 M or 1.3 M sucrose and subsequently transferred to lower osmolarities. The granules either were transferred back in one step (○, □) or step by step (+) to the sucrose concentration indicated.

for 1 h and then transferred to 0.52 or 0.39 M sucrose, respectively, either directly in one step or in 20 steps, lasting altogether for 20 min. There was only a negligible difference between these methods.

To investigate the influence of the incubation time on hyperosmotic relaxation lysis granules were incubated in 0.78 M buffered sucrose solution for different periods and then transfered to solutions of lower osmolarities, see Fig. 2. There was a slight loss (4%–13%) of catecholamines after 1 or 1.5 h incubation in 0.78 M sucrose solution. On decreasing the external osmotic pressure hyperosmotic relaxation lysis occurred, and this was enhanced with increasing incubation times. However, the shortest possible incubation time of 1 min at high osmolarities was already sufficient to cause irreversible alterations of the granules leading to hyperosmotic relaxation lysis (Fig. 2).

Size measurements by means of photon correlation spectroscopy

To obtain information about the lysis mechanism at high osmolarities, the sizes of granules were measured. Fig. 3 shows mean ($1/z$ average) diameters of chromaffin granules from four different preparations as a function of sucrose concentration. Full circles represent mean diameters of granules after 10 min incubation at the respective osmolarity. The mean diameter of granules decreased on increasing the external osmotic pressure. When the volume of granules was calculated from these data, assuming a spherical shape, and plotted vs. the inverse osmotic pressure a straight line was obtained (not shown). This indicated the behaviour of an ideal osmometer reported by Morris et al. [3] and Südhof [1].

The other symbols in Fig. 3 refer to granules preincubated in 0.78 or 1.3 M sucrose solution and transferred back to lower sucrose concentrations indicated. Surprisingly, with decreasing osmotic pressure the mean size of lysing granules increased monotonically and continuously. Within the error of this measurement, no significant difference between both populations of granules, intact and lysing, could be observed. Thus, a simple lysis mechanism caused by a permeability increase for the osmoticum sucrose, its uptake, water influx and subsequent lysis, can be excluded. In such a case, the granules lysis at hypertonic osmolarities should have a diameter as large as or even larger than that at isoosmotic pressure.

Membrane labelling experiments

In order to determine whether there was a loss of membrane under hypertonic conditions by endocytotic uptake into the granules, EPR- and fluorescence-labelling experiments were performed. Both probes, ONS and CAT 16, should label only the outer leaflet of granule membranes [27,29]. If excess membrane was driven into the granules and separated from the outer membrane during osmotic shrinkage one would expect to obtain a signal from label in the inner membrane compartment even after destruction of the labels located in the outer granule membrane.

TNBS reacts with amino groups of lipids and membrane proteins forming a fluorescent reaction product [28]. As its absorption spectrum overlaps with ONS emission spectrum it causes quenching of ONS fluorescence, cf. [27]. In our experiments, reaction with TNBS quenched the fluorescence of ONS in control granules (in isotonic sucrose) from 100% to 60%. The same value was obtained for labelled granules which were incubated in 0.78 M sucrose and to which TNBS was added after osmotic shrinkage.

In EPR experiments, the spin label located in the granule membrane should be reduced by ascorbic acid. Ascorbic acid itself does not permeate membranes at 4°C [33]. Since chromaffin granules are known to have several membrane-bound and inner redox systems [26] we studied their activity in a parallel experiment using the water-soluble spin probe TEMPO. After 45 min the spin signal of 0.1 mM TEMPO added to the granule suspension only decreased from 100% to 91%. Thus, the redox activity of the granules themselves was negligibly low at 4°C.

After addition of 1 mM ascorbic acid to labelled control granules (in isotonic buffered sucrose) the CAT 16 signal was completely destroyed. The same result was observed for labelled granules which were incubated in 0.8 M sucrose solution before the addition of ascorbic acid. Given that neither TNBS [27] nor ascorbic acid [29,33] penetrated the granule membranes, inwardly-directed pinching off of membrane can be excluded. Suspensions of CAT 16-labelled and hyperosmotically treated granules were centrifuged at $12000 \times g$ for 12 min. The supernatant did not show any spin signal

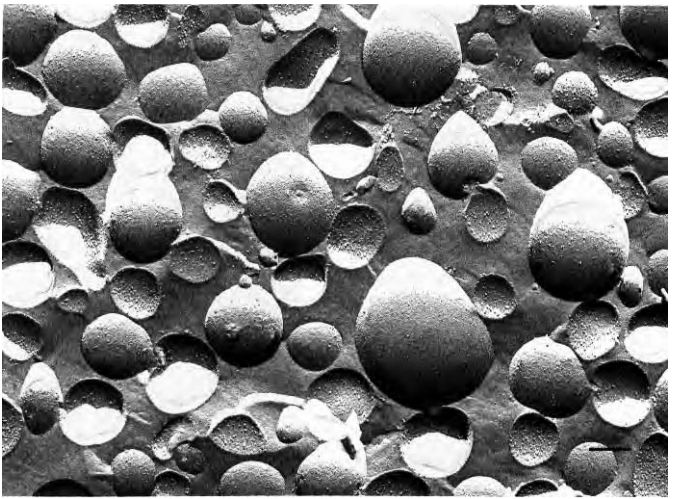

Fig. 4. Isolated chromaffin granules in isotonic sucrose solution. In all micrographs the bar represents 200 nm and the shadow direction is from bottom to top.

which suggested that the granules did not vesiculate into small separated parts either.

Freeze-fracture electron microscopy

For the direct observation of these phenomena by electron microscopy, we used the freeze-fracture technique. Here we show micrographs of unfixed granules only. Fixation with 2% glutaraldehyde led to artifacts such as crinkled membranes and cluster formation of intramembranous particles (IMPs) even for controls. In general, however, similar characteristic alterations of hyperosmoticaliy treated granules were observed in glutaraldehyde-treated membranes as in unfixed samples.

Isolated chromaffin granules suspended in isotonic buffered sucrose were found to be dispersed (Fig. 4). Most of them were of spherical shape with a mean diameter of approx. 400 nm which was in agreement with the data obtained by photon correlation spectroscopy (Fig. 3). The concave fracture faces with more IMPs are the P-faces (protoplasmic fracture faces), the convex fracture faces with less IMPs the E-faces (exoplasmic fracture faces). Intramembranous particles were distributed randomly. Generally, cross fractures of granules were found rarely (Fig. 5) which is a common effect for vesicles of this size. Part of cross-fractured granules revealed 1 to 2 intragranular vesicles. These vesicles were round to oval and had a size of 50–200 nm diameter (Fig. 5b–d).

On increasing the osmotic pressure by the addition of sucrose (0.5–1.2 M) the mean diameter of granules decreased, see also Fig. 3. Many granules were transformed into 'twin'-shaped granules or even 'triplets' (Fig. 6). These deviations from sphericity were already found in granules suspended in 0.5 M sucrose solutions, but the parts of the 'twins' were less pronounced. In some cases the fracture path had left the membrane of the granule exposing membranes of internal vesicles (Fig. 6a, arrow). Apposed intragranular vesicles can be clearly distinguished by the cleft between them whereas the covering granule membrane is devoid of such a cleft. In some cases, deformations of the granules resembled 'bubbles' placed underneath the granule membrane (Fig. 6b). Additionally, the distribution of intramembranous particles was no longer random. Often the larger part of the 'twin' showed the majority of the particles, whereas the other part was almost free of them (Fig. 6a).

Cross fractures of granules were rare, but they also showed intragranular vesicles (Fig. 7a–d). One to five

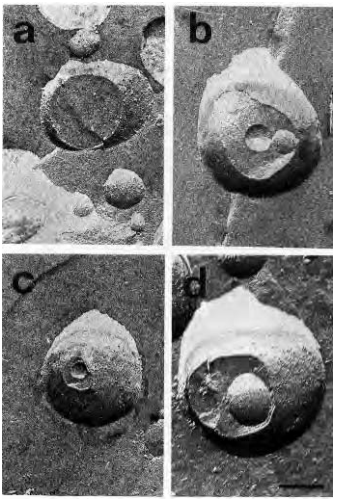

Fig. 5. Cross-fractured chromaffin granules in isotonic sucrose solution revealing no (a), small (b, c) or large (d) intragranular vesicles.

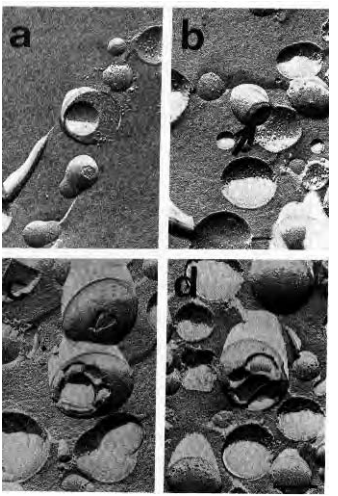

Fig. 7. Cross-fractured chromaffin granules after hypertonic incubation revealing one (a) or two (b), but also more (c, d) intragranular vesicles. In (b) only one intragranular vesicle is still visible in the 'twin', but the flattened contact area (arrow) suggests on a second vesicle broken away.

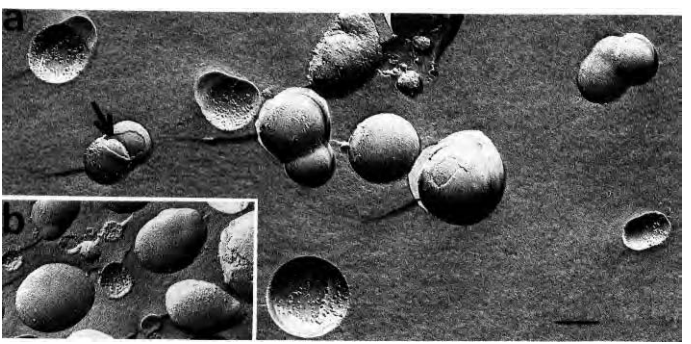

Fig. 6. Chromaffin granules after hypertonic incubation in 1.2 M (a) or 1.0 M (b) sucrose solution. Nonspherical granules, mostly 'twins' and 'triplets', are obviously deformations induced by intragranular vesicles (arrow).

intragranular vesicles were observed in these granules, the larger ones had a diameter of about 150 nm, the smaller ones a diameter of 50–90 nm. In general, intragranular vesicles found in hyperosmotically shrunken granules appeared to have the same size as in isotonically suspended granules (Fig. 5). Often intragranular vesicles seemed to be pressed together and towards the granule membrane since many of them were not spherical (Fig. 7b–d).

Discussion

Hyperosmotic lysis behaviour of chromaffin granules is shown in Fig. 1. Data from this figure were used to construct Fig. 8, in which the sucrose concentration range $\Delta c_{50\%}$ over which granules had to be transferred to yield 50% catecholamine release is plotted vs. the incubation concentration. Open and closed circles represent two different granule preparations. The osmotic tolerance $\Delta c_{50\%}$ increases almost linearly with the sucrose concentration in the incubation.

To interprete this behaviour let us consider two limiting cases. Curve 1 in Fig. 8 represents ideal reversible behaviour of granules. A value of 180 mM sucrose was assumed for one-half maximal lysis independent of preincubation at elevated osmolarities. The other limiting case is irreversible shrinkage of granules accompanied by loss of excess membrane (curve 2). Moving the granules back to lower osmolarities we assume that they would stand a decrease of external sucrose concentration of 80 mM at most. This value was taken from hypoosmotic lysis experiments assuming that the critical membrane tension for lysis $\Delta S = 21$ mN/m (calculated from the law of Laplace, see below) is a constant. Experimental data fall between these limiting curves.

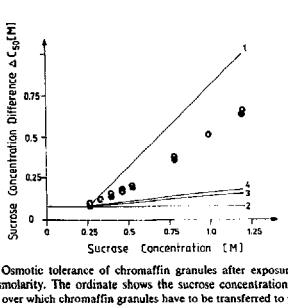

Fig. 8. Osmotic tolerance of chromaffin granules after exposure to hyperosmolarity. The ordinate shows the sucrose concentration difference over which chromaffin granules have to be transferred to yield 50% catecholamine release as a function of the incubation concentration. ○, ●, experimental data from two granule preparations. Curves 1–4 are theoretical curves. Curve 1 demonstrates the lysis behaviour of reversible shrunken granules if they behaved as ideal osmometers. One-half maximal lysis was assumed to occur at 180 mM sucrose. Curve 2: hypothetic lysis behaviour of irreversible shrunken granules that have lost excess membrane. A critical sucrose concentration difference of 80 mM, corresponding to a critical membrane tension of 21 mN/m, was assumed to induce lysis. The radius decrease upon shrinkage, which was neglected for curve 2, was taken into account for curve 3 to calculate osmotic resistance from the law of Laplace. The same was done for curve 4, in which the parts of the 'twins' were considered to be separated.

In curve 2, the radius decrease caused by osmotic shrinkage was neglected. However, according to the law of Laplace $\Delta S = r\Delta\pi RT/2$ (r = radius of the granule, π = osmotic pressure), small granules have a higher osmotic resistance than large ones. Taking into acount the radius decrease in hypertonic solutions, see Fig. 3 and Ref. 1, curve 3 is obtained. If the granules of 'twinned' shape observed by electron microscopy consisted of two or more internally separated compartments their osmotic resistance should be calculated from the radii of the single parts as shown in curve 4. Neither curve 3 nor curve 4 fit the experimental data.

From these considerations it can be concluded that either granules incubated in hypertonic solutions consist of two sharply different populations one behaving completely reversible, the other irreversible, cf. curves 1 and 2 in Fig. 8. The other possibility is that the granules are homogeneous and undergo incomplete irreversible osmotic shrinkage (cf. case 2).

From the EPR- and fluorescence-labelling experiments we exclude membrane loss by pinching off of membrane material into the granules or into the bulk solution. By means of freeze-fracture electron microscopy twin or triplet granules formed at high osmolarities were found. The question then arises as to the nature of these structures.

'Twin' formation was observed for chromaffin granules [34] and other secretory vesicles (vesicles from the islet of Langerhans [35], Golgi-derived vesicles from rat liver [36], neurosynaptosomes [37], vesicles formed from myoblast membranes [38]) after addition of 0.01–1 mM Ca^{2+} which caused aggregation and fusion [34–38]. Though the 'twins' observed in our study resemble these aggregated or fused vesicles, at least two arguments show that they were not caused by aggregation or fusion of individual granules. Firstly, size measurements contradict such a model since the size of granules decreased continuously and monotonically with increasing osmotic pressure (Fig. 3). Secondly, 'twin' formation and hyperosmotic relaxation lysis itself depended on incubation osmolarity as a continuous function. Moreover, Fig. 6b suggests that the 'twins' were generated by granule membrane deformations which arose from the granule interior and that they were no aggregation or fusion products of individual granules.

Our observations are more in accordance with the interpretation that 'twins' and 'triplets' were induced by the attachment of intragranular vesicles to the gran-

ule membrane. Probably this process also induced the separation of IMPs from the granule membranes within the areas of contact to intragranular vesicles.

Recently similar small (50–150 nm in diameter) vesicles have been found by Ornberg and co-workers [22] inside isolated and non-isolated chromaffin granules after quick freezing and freeze substitution. As these vesicles are labile to fixation with aldehydes and following dehydration they have been detected only by using physical fixation. One to five membrane-bounded, electron-translucent vesicles were observed in 60–85% of all granules. Neither the contents nor functions of intragranular vesicles are known at present [22].

Using the freeze-fracture technique, though not frequently, cross fractures of granules were obtained. They revealed intragranular vesicles under both, isotonic (Fig. 5b–d) and hypertonic (Fig. 7) conditions. Their size (50–150 nm) was almost unaffected by an increase of osmotic pressure in agreement with the findings of Ornberg et al. [22].

Intragranular vesicles were not detectable from the outside in isotonically suspended granules. Its local curvature and particle distribution did not indicate the presence of intragranular vesicles underneath the granule membrane. Repulsive forces or other mechanisms obviously prevented intragranular vesicles from exerting any detectable influence on the granule membranes. What might be the reason for the strong granule deformation at hypertonic conditions?

Even under isotonic conditions the granule core is a very concentrated solution (0.5–0.7 M catecholamines, 0.12 M ATP, ions, proteins and neuropeptides [8,25,26]) showing nonideal osmotic behaviour. The osmotic pressure of this solution is not equal to the theoretical value of 0.8 osM. Instead it is equal to only 0.3 osM mainly due to catecholamine–ATP interactions [40,41].

With increasing osmotic pressure the granule core becomes more and more dehydrated. The micrographs (Figs. 6 and 7) show that dehydration goes along with a close approach of the inner surface of the granule membrane and intragranular vesicles.

Dehydration should result in an increase in ionic strength, reducing electrostatic repulsion between adjacent charged surfaces. Since hydration forces are of major importance in stabilizing macromolecular solutions and interfaces [39] the osmotically-induced dehydration of the granules may also directly influence the stability of the granule contents. One explanation is that the core material of the granule upon dehydration excludes the intragranular vesicles. It is also possible that dehydration of both the intragranular vesicle surface and the granule membrane surface directly favours mutual adhesion, since the hydration of surface groups is known to be the major reason for membrane-membrane repulsion at short distances [39].

However, we clearly see that independent of the initial cause of the close approach of the vesicles and the granule membrane structural changes of the granule membrane become apparent. We see depletion of IMPs and well pronounced curvature changes.

It is our hypothesis that these changes indicate a gain in interfacial free energy from adhesion and do not simply result from squeezing the vesicles towards the inner surface of the granule membrane. This hypothesis is in accordance with the main experimental result of hyperosmotic relaxation lysis. The mechanism of this process might be the following: Upon decreasing the osmotic pressure after hypertonic incubation the vesicles separate more slowly from the granule membrane than water enters the granule. This is equivalent to a reduced apparent surface to volume ratio of the granules, because the regions of the granule membranes attached to the vesicles remain highly curved. Their adhesion energy must be large enough to prevent peeling off due to tangential stress created by swelling.

This mechanism does not require irreversible attachment of both membranes. Kinetic irreversibility in the time scale of swelling should be sufficient since the swelling is very rapid for the given small size of granules. There might be not enough time for the vesicles to wait for separation upon rehydration.

Another question is whether this mechanism has some significance in explaining the inhibition of secretion at high osmotic pressures. In both intact [5,13,14] and permeabilized [5,8,9,13] chromaffin cells, secretagogue- and calcium-induced exocytosis, respectively, is inhibited by hyperosmolar solutions at a step after calcium entry [9,14]. The inhibitory effect of hyperosmolar sucrose solutions on secretion from permeabilized cells [5,9,13] has been shown to be less effective than on that from intact cells [9,13,14]. This difference in the secretory response could result from an increase in the ionic strength [9] or an increase in intracellular pH [14] induced by osmotic shrinkage of intact cells. It has been demonstrated that elevated ionic strength as well as elevated pH partly inhibits exocytosis [9,14]. In contrast, inhibition of exocytosis in permeabilized, hypertonically suspended cells could be in part due to a loss of proteins essential for exocytosis [42].

The existence of intragranular vesicles demonstrated first by Ornberg et al. [22], and especially the strong interaction of these vesicles with the granule membrane demonstrated herein, require to consider the possibility that the inhibition of exocytosis by hyperosmolar solutions might also be caused by intragranular vesicle–granule membrane interaction. It is thought that swelling of the granules is crucial for completing exocytosis but not necessary for intiating it [11,12]. Consequently, if the strong interaction of intragranular vesicles with the granule membrane is the cause of hyperosmotic relaxation lysis, it may also be expected partly to prevent the swelling process of the granules, such that

release of the contents might be hindered. Another possibility is that the IMP-free regions of the granules observed are less capable to fuse with the plasma membrane [43].

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