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Ultrastructural and cytochemical characterization of adrenal medullary plasma membrane vesicles and their interaction with chromaffin granules

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Plasma membrane vesicles obtained by density gradient centrifugation of bovine adrenal medullary homogenates were analyzed by electron microscopic methods, including negative staining, ultrathin sections and freeze-fracture replicas. Rapid freezing showed the intramembrane structure of plasma membrane vesicles to be distinct from that of other organelle membranes, such as chromaffin granules. Cytochemical demonstration of acetylcholinesterase (EC 3.1.1.7) activity on most membrane profiles confirmed that plasma membrane vesicles are derived predominantly from plasma membranes. About half of the plasma membrane vesicles were smaller than 0.15 μm and almost none larger than 0.55 μm. Practically all were composed of single shells. Most vesicles were impermeable to cytochemical markers of the size of Ruthenium red (M_r 800) and none were permeable to markers larger than 40 kDa. Surface charge probes, concanavalin A binding and endogenous actin decoration with heavy meromyosin indicated that the major fraction of plasma membrane vesicles is oriented right-side-out. A minor population with opposite orientation could also be detected. Isotonic ionic media caused vesicle aggregation in suspensions of plasma membrane vesicles and chromaffin granules. Freeze-fracturing always revealed clusters of membrane-intercalated particles at the sites of contact between aggregated membranes.

Introduction

The limit requirements of exocytosis are difficult to ascertain within intact cells. Essentially three different attempts have been made to solve this problem: (a) Complexes containing plasma membranes with secretory organelles attached were isolated; this is possible only in systems with permanent firm links between these components, such as oocytes [1], and, thus, cannot be applied to adrenal chromaffin cells. (b) Cells were rendered leaky by a high voltage discharge [2], or by detergent treatment [3–5]; this still represents a rather complex system. Most of the macromolecular components are retained within the cell, and secondary processes, like exocytosis-coupled endocytosis still can take place. Another approach was to isolate plasma membrane vesicles and to attempt reconstruction of a secretory system using the secretory organelles and these vesicles. This last approach to analyze exocytosis in vitro with fractions of adrenal medullary homogenates, was made by several groups [6–10]. The plasma membrane vesicles used in these experiments had been characterized mainly biochemically [10,11], and it appeared, thus, necessary to correlate the biochemical data with ultra-

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Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate; F-actin, filamentous actin; IF, inner fracture face; OF, outer fracture face.

structural information on size, purity, sidedness and leakiness. The ultrastructural organization of the chromaffin secretory granule, in situ and after isolation, in ultrathin sections [12] and in freeze-fracture replicas [13–15], has already been well established and it could, therefore, also be attempted to analyze ultrastructural features of the interaction between plasma membrane vesicles and chromaffin granules.

Materials and Methods

Isolation of plasma membrane vesicles. Plasma membranes were prepared according to Wilson and Kirshner [11] with slight modifications. Bovine adrenal glands from freshly slaughtered cattle were put on ice and all subsequent steps were carried out on ice or at 4°C. The medullae were separated from the cortices, finely cut and homogenized in 0.3 M sucrose, 0.01 M Tris-HCl (pH 7.0) (approx. 1 g tissue per 5 ml) using a Duall, glass to glass, tissue grinder. The homogenate was centrifuged at $1200 \times g$ (Sorvall SS-34 rotor) for 10 min and the supernatant centrifuged at $20000 \times g$ for 30 min. The pellet was suspended in the sucrose-Tris buffer, layered over 1.4 M sucrose, 0.01 M Tris-HCl (pH 7.0), and centrifuged at $100000 \times g$ for 90 min (Beckman SW 28 rotor). The material of the interphase was collected, diluted 3-fold with 0.01 M Tris-HCl and centrifuged at $64000 \times g$ for 30 min (Beckman Ti 60 rotor) to pellet the membranes. The pellet was resuspended as before, layered over 11% (w/v) Urografin solution in 0.3 M sucrose, 0.01 M Tris-HCl (pH 7.0) and centrifuged at $100000 \times g$ for 120 min (Beckman SW 28 rotor). The material from the interface was collected, diluted at least 4-fold with the sucrose-Tris buffer and pelleted by centrifugation at $64000 \times g$ for 30 min (Beckman Ti 60 rotor). Pellets were stored at −80°C until used. The acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) activity of this preparation assayed according to Ellman et al. [16], ranged from 0.6 to 0.8 μmol thiocholine formed/min per mg protein. As was shown before by Schneeweiss et al. [17], this membrane fraction has very low dopamine-β-hydroxylase (EC 1.14.17.1) and monoamine oxidase (EC 1.4.3.4) activities, indicating that contamination by membranes derived from either mitochondria or chromaffin granules is low.

Urografin was supplied as a 60% aqueous solution by the Schering A.G., Berlin, Ficoll 400 by Pharmacia, Fine Chemicals, Uppsala.

Chromaffin granules were isolated by the isotonic density gradient method of Trifaró and Dworkind [18]. They were stored on ice in 0.3 M sucrose.

Actin was prepared from rabbit back and leg muscle, as described by Spudich and Watt [19] and heavy meromyosin was obtained from the same source according to the procedure of Weeds and Pope [20]. The proteins were stored at 4°C and used within 3 weeks. The association of actin with the plasma membrane vesicles was studied, using the actin binding buffer described by Wilkins and Lin [21]. For decoration of endogenous or exogenous actin with heavy meromyosin, the latter was applied in a buffer composed of 40 mM NaCl, 5 mM phosphate (pH 6.5) and 0.1 mM NaN_3. Plasma membrane vesicles and equal amounts (w/w) of either one or both of these proteins were incubated at room temperature for 3 h, the plasma membrane vesicles were pelleted by centrifugation at 18000 rev./min for 10 min (Beckman model J-21, rotor JA 20) and processed for electron microscopy.

Protein was determined by the method of Bradford [22].

The interaction of plasma membrane vesicles with chromaffin granules was studied by incubating equal amounts (of protein) of the two components (100–500 μg) for 10 min in either 0.3 M sucrose-0.01 M Tris (pH 7.0), or 2 mM Ca^{2+} containing isotonic salt media: (1) 120 mM KCl, 30 mM NaCl, 0.01 M Tris (pH 7.0); (2) 150 mM potassium glutamate, 2 mM EGTA, with or without 5 mM Mg-ATP. Incubation was stopped by 2.5% glutaraldehyde in ice-cold 0.3 M sucrose solution.

Electron microscopy. For analysis by standard ultrathin sectioning techniques samples were fixed in 1.5% glutaraldehyde and subsequently in 2% OsO_4, all in 0.1 M cacodylate buffer (pH 7.0), followed by acetone dehydration and embedding in Spurr's resin. Sections were stained with 2% aqueous uranyl acetate and alkaline lead citrate. This procedure was used also in conjunction with different cytochemical staining reactions.

Occasionally permeabilization of plasma mem-

brane vesicles preparations was achieved by adding 0.01–0.1% saponin which produces holes of approx. 5 nm diameter [23].

Staining by concanavalin A and peroxidase (Sigma type VII) was according to Bernhard and Avrameas [24], Ruthenium red staining followed the recipe by Luft [25] and staining with cationic (or native) ferritin that by Skutelsky and Danon [26]. Peroxidase isoenzyme preparations of different p*I* (type VII and IX from Sigma) were visualized according to Graham and Karnovsky [27]. All these probes were applied at pH 7.0.

For the visualization of F-actin tannic acid was applied as a mordant dye [28], followed by section staining (see above).

Acetylcholinesterase activity was visualized by the method of Satoh et al. [29] using acetylthiocholine iodide as a substrate.

For negative staining samples were briefly fixed with 1.5% glutaraldehyde, washed in distilled water and suspended in 1.5% phosphotungstic acid.

Cryofixation of samples incubated at either room temperature or 40°C, was done by the sandwich-propane-jet procedure according to Pscheid et al. [30] and Knoll et al. [31]. Only for deep-etching experiments were the samples fixed with 2.5% glutaraldehyde and briefly washed in distilled water before rapid freezing.

Freeze-fracturing was carried out at −100°C in a Balzers BAF 300 device equipped with a double fracture holder.

Electron microscopic evaluation was performed with a Zeiss EM10 with calibrated magnification steps.

Results

Ultrastructural evidence for enrichment of plasma membranes

Several criteria were used to assess the purity of the plasma membrane vesicles: (a) Fragments of mitochondria, secretory granules or other identifi-

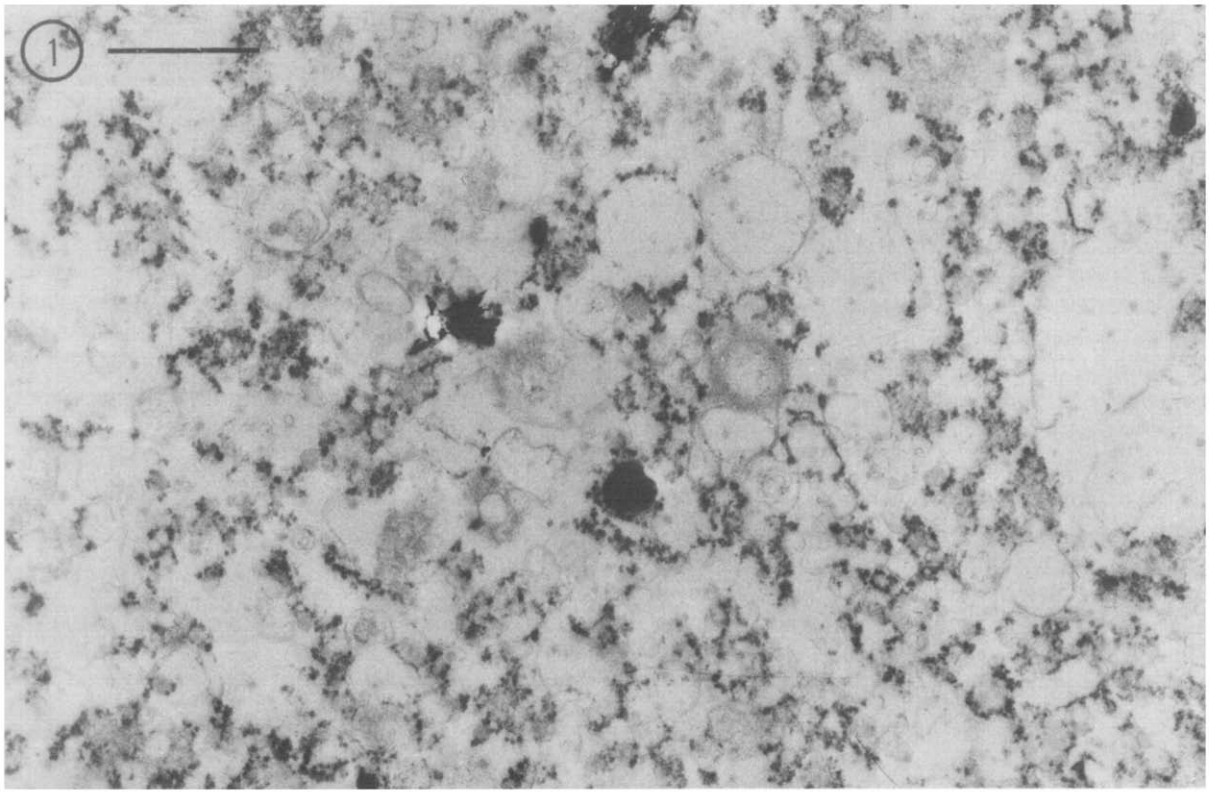

Fig. 1. Plasma membrane vesicles in an ultrathin section with a cytochemical demonstration of acetylcholinesterase activity. Bar = 1 µm.

Fig. 2. Freeze-fractured plasma membrane vesicles showing scarcity of membrane-intercalated particles on IF and OF aspects as clustering of membrane-intercalated particles. Bar = 0.1 μm.

able subcellular structures were practically not recognized in the plasma membrane vesicles fraction, regardless of whether we analyzed ultrathin sections or negatively stained preparations (data not shown). (b) Using the cytochemical acetylcholinesterase assay, most membranes were found to be reactive (Fig. 1). (c) The appearance of freeze-fracture micrographs was homogeneous and distinct from that of chromaffin granules or the membranes derived from these (Fig. 2). While the membranes of chromaffin granules do not exhibit clustering of membrane intercalated particles even when fragmented [15] the preparations examined in the present work showed extensive clustering under all the conditions used.

Size distribution

This could be estimated from freeze-fracture replicas, but does not reveal the real diameter, since for vesicle populations comprising different type classes the data are difficult to correct for random fracturing. Therefore, apparent size classes are presented in Table I, which shows that approx. 50% of plasma membrane vesicles are not larger than 0.15 μm and approx. 85% are not larger than 0.25 μm, close to the size of chromaffin granules, i.e. approx. 0.22 μm. Ultrathin sections and negatively stained preparations gave essentially identical results.

TABLE I

APPARENT SIZE DISTRIBUTION OF PLASMA MEMBRANE VESICLES, DERIVED FROM FREEZE-FRACTURE REPLICAS

Size class (diameter (μm))	Frequency (%)
$\varnothing < 0.15$	54
$0.15 < \varnothing < 0.25$	31
$0.25 < \varnothing < 0.55$	13
$0.55 < \varnothing$	2

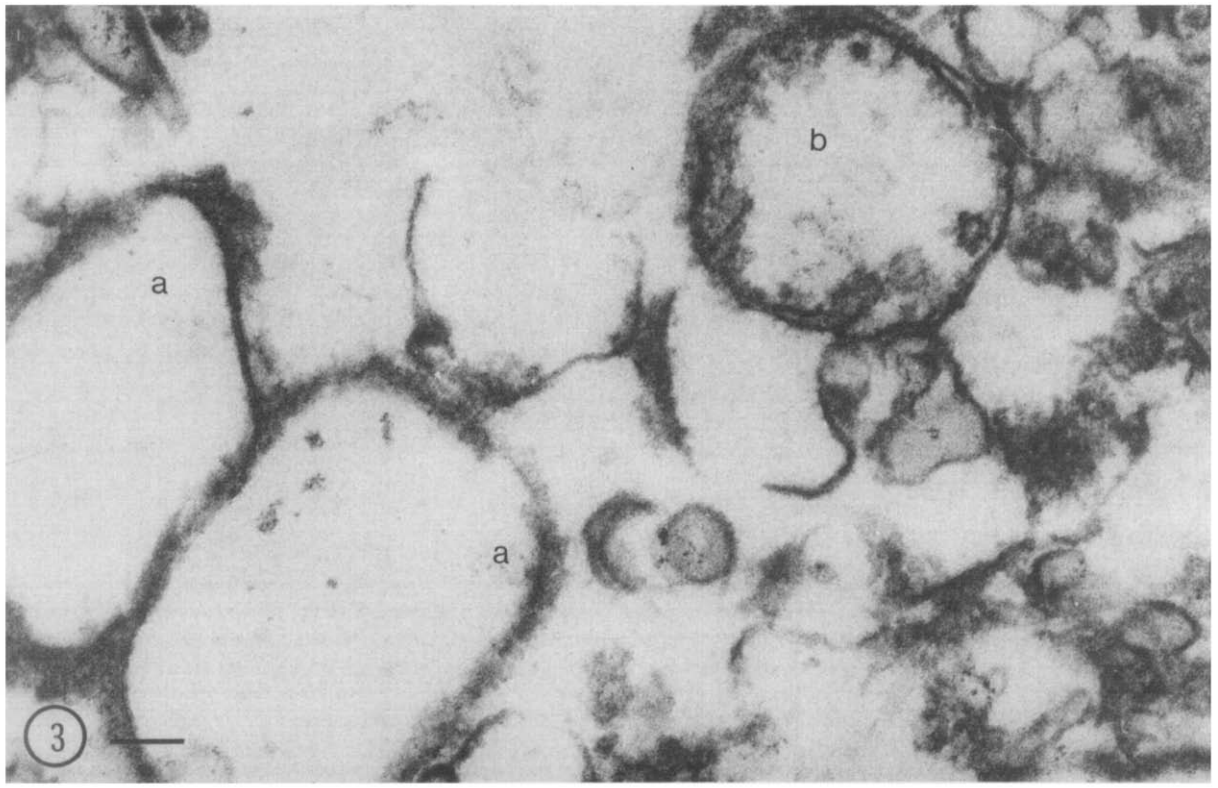

Fig. 3. Plasma membrane vesicles permeabilized with 0.01% saponin, incubated with concanavalin A and stained with horseradish peroxidase VII and 3,3′-diaminobenzidine. A major fraction of plasma membrane vesicles would appear stained on the outer surface (e.g., in (a)), only a minor fraction on the lumenal side (e.g., in (b)). Bar = 0.1 μm.

Number of layers

All techniques used, i.e. freeze-fracturing, ultrathin sectioning and negative staining revealed that most plasma membrane vesicles have one shell. Multiple membrane layers do occur, though rarely, also in rapidly frozen freeze-fracture samples, and some plasma membrane vesicles also contain small vesicles inside.

Leakiness

Without saponin permeabilization only a minor fraction of large or small plasma membrane vesicles were accessible to horseradish peroxidase (M_r 40000, diameter approx. 4.5 nm; for size and charge of electron markers, see Ref. 23). Larger probes, like ferritin, both anionic and cationic ($\varnothing \sim 11$ nm) could not enter the plasma membrane vesicles; neither could heavy meromyosin (M_r 350000). The size of the holes in this permeable fraction would, therefore, be approx. 5 nm, and not more than approx. 10 nm. This could explain the relatively small pool of membrane associated F-actin that was found to be sensitive to digestion by DNAase I, (M_r 31000 [10]), even though it is localized on the inside of the plasma membrane vesicles. (For some additional F-actin on the outer surface of the plasma membrane vesicles, see below). Similar to horseradish peroxidase, Ruthenium red (M_r 800 [25]) was found to enter only a small number of vesicles, and it can thus be concluded that most of the plasma membrane vesicles are tightly sealed.

Membrane structure after freeze-fracturing

In plasma membrane vesicles (Fig. 2) the density of membrane-intercalated particles was similar in the IF * fractures and in the OF * fractures,

* IF and OF designate the inner (convex) and outer (concave) fracture. We use this nomenclature since the actual anatomical orientation of individual plasma membrane vesicles is not known.

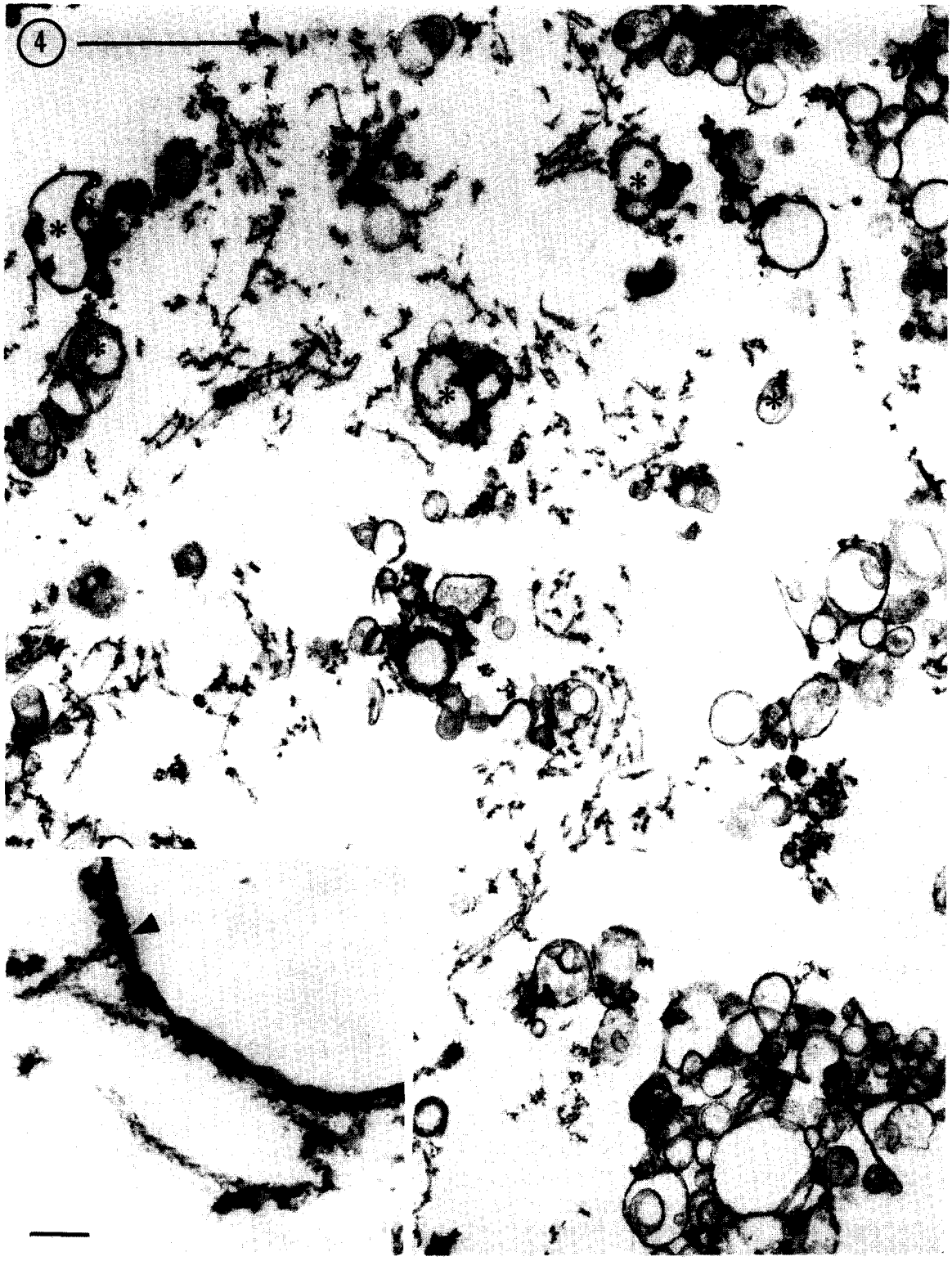

Fig. 4. Plasma membrane vesicles incubated with actin for F-actin formation and heavy meromyosin decoration. Only a minor proportion of plasma membrane vesicles can thus be labeled (asterisks). Beyond this, this figure shows some variability in size, the absence of larger amounts of identifyable contaminants, the occurrence of a single shell organization in most cases, and the occasional occurrence of smaller vesicles within plasma membrane vesicles. Bar = 1 μm (inset, 0.1 μm).

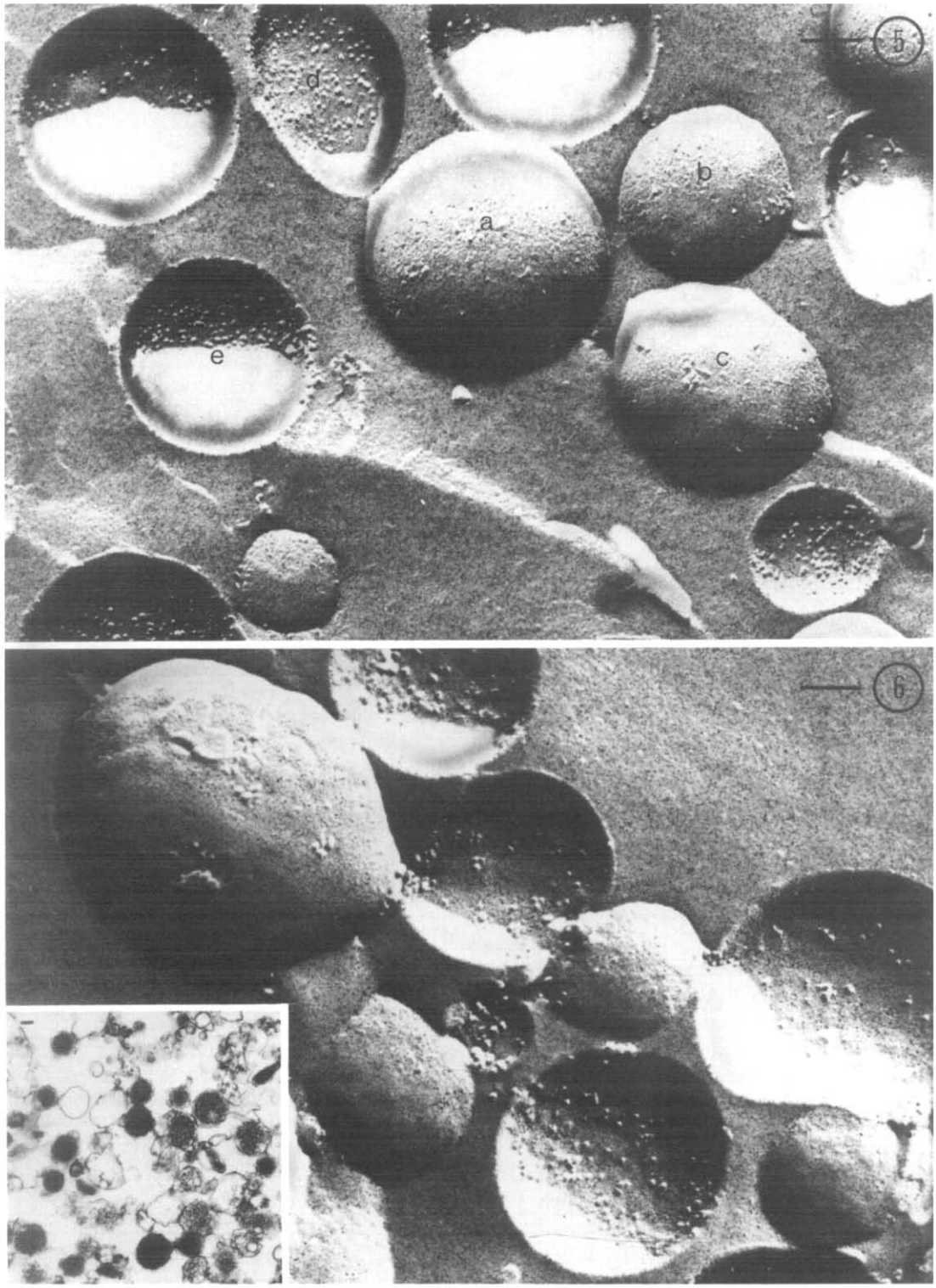

Fig. 5. Mixture of plasma membrane vesicles and chromaffin granules (1 : 1) in sucrose. Vesicles can be identified bona fide as plasma membrane vesicles by rare membrane-intercalated particles in clusters (a, b, c), or as chromaffin granules by abundant loosely scattered membrane-intercalated particles (d, e, f). No vesicle clustering occurs. Bar = 0.1 μm.

Fig. 6. Same as in Fig. 5 but incubated in high ionic media to induce vesicle clustering (see Materials and Methods) which occurs at sites of membrane-intercalated particles clustering. The inset shows the occurrence of PMV-PMV, CG-CG and PMV-CG clustering. Bars = 0.1 μm. PMV, plasma membrane vesicle; CG, chromaffin granule.

and much lower than that of the membrane-intercalated particles in chromaffin granules, particularly that on their concave (OF) face, i.e. 950/μm^2 [14]. Furthermore, in contrast to chromaffin granules, where membrane-intercalated particles cluster only under very special conditions [15], those of plasma membrane vesicles, suspended in various ionic media, were always clustered on either fracture face, regardless of vesicle size, temperature (0° to 40°C), as well as the presence or absence of exogenous actin (see below). Clustering of membrane-intercalated particles did also not depend on the aggregation of plasma membrane vesicles. The similar density of membrane-intercalated particles displayed by the two fracture faces made it impossible to ascertain sidedness of the plasma membrane vesicles, using freeze-fracture micrographs.

Plasma membrane vesicle sidedness from cytochemical marker experiments

Ruthenium red, an electron stain, stains mainly the outside and, after permeabilization, also most of the inside of the plasma membrane vesicles. Concanavalin A was expected to provide a clearer assignment of sidedness (Fig. 3). Most plasma membrane vesicles, when analyzed without permeabilization, had a faint coat indicative of glycocalix elements, while, after permeabilization, this could be found sometimes also on the inner side. Controls with 3,3′-diaminobenzidine alone gave no staining reaction. The reaction product of acetylcholinesterase activity (Fig. 1) was found to be located mainly on the outer surface of the plasma membrane vesicles and a large proportion of plasma membrane vesicles was reactive. These two tests indicate that only a minor fraction of the plasma membrane vesicles is oriented inside-out.

Sidedness from actin and heavy meromyosin binding

Tannic acid stainable filamentous structures were recognized within some plasma membrane vesicles, whereas some others displayed such material on their outside. This could represent cytoskeletal, but also glycocalix elements. When exogenous actin was added to permeabilized plasma membrane vesicles in the actin polymerizing medium, many of these showed filamentous material in their interior upon tannic acid treatment. Only in a minor fraction could actin filament formation be induced on the outer side (Fig. 4). Upon decoration with heavy meromyosin, the arrowheads pointed almost always away from the membrane (inset in Fig. 4).

On freeze-fracture replicas of heavy meromyosin-treated plasma membrane vesicles (data not shown) some of the surfaces appeared studded with heavy meromyosin. This could be due to a diffuse covering with small F-actin chains. In the presence of exogenously added actin, strands of 10 to 20 nm length were observed, but neither these, nor bound heavy meromyosin, were in any way restricted to or excluded from regions of membrane-intercalated particles clustering.

Freeze-fracturing of plasma membrane vesicles and chromaffin granule interaction

When plasma membrane vesicles and chromaffin granule fractions were combined in 1 : 1 proportion in 0.3 M sucrose, no visible interaction took place (Fig. 5). However, when similar experiments were conducted in high ionic strength media, both elements frequently combined into small aggregates (Fig. 6). Whenever membrane contact occurred, it was at regions where membrane-intercalated particles appeared in clusters, at least on one of the two membranes involved. Whether fusion at these contact sites was actually taking place could not be ascertained by the present observations.

Discussion

Using ultrathin sectioning, in conjunction with cationic electron stains or cytochemical markers, we have characterized the PMV with respect to purity, inside-out vs. right-side-out orientation and leakiness. Additional supportive information was obtained by freeze-fracturing after cryofixation, through the use of actin-binding and heavy meromyosin decoration. In freeze-fracture micrographs plasma membrane vesicles can be easily distinguished from chromaffin granules, both by the number and the extensive clustering of membrane-intercalated particles on the fracture faces of the former. Notable is the scarcity of identifiable contaminants, which is in line with previous measurements of enzyme markers, showing very low contaminations by chromaffin granules or

mitochondria [11,17]. The latter would be recognized, particularly in negatively stained preparations, by their numerous membrane attached particles. The acetylcholinesterase activity of the plasma membrane vesicles used in our measurements ranged from approx. 600 nmol to approx. 800 nmol thiocholine formed per min per mg protein, some 20- to 50-times higher than values recorded for this enzyme activity in chromaffin granules [32,33]; thus, the potential contribution of granules in our cytochemical acetylcholinesterase assay would be negligible, and most chromaffin granules, if present as impurities, would appear unstained. In sum, the procedure applied in the present work yields a very considerably enriched fraction of plasma membranes.

The sidedness of plasma membrane vesicle fractions, obtained by the same fractionation protocol has been assessed previously by α-bungarotoxin binding, acetylcholinesterase activity and measurement of membrane associated creatine kinase (EC 2.7.3.2) [10]. While approx. 95% of α-bungarotoxin binding sites, as well as of acetylcholinesterase activity, were accessible without previous Triton X-100 digestion, approx. 80% of the creatine kinase activity was found to be latent. A quantitatively similar latency was found for endogenous actin. These values indicated that most of the plasma membrane vesicles were right-side-out and that at least 80% of these were not leaky. The pool of F-actin that was accessible to DNAase I digestion could have been due partly to leakiness and partly to the small fraction of plasma membrane vesicles with inside-out orientation. The ultrastructural data presented here are in line with these conclusions. The inside-out plasma membrane vesicles show up in both ultrathin sections and freeze-fracture micrographs prepared from vesicles to which exogenous actin was added under polymerization conditions, followed by decoration with heavy meromyosin. This aspect is supported also by the concanavalin A staining experiments, showing a small fraction of plasma membrane vesicles with marker on the inner surface.

The predominantly right-side-out orientation of the plasma membrane vesicles is a potentially major drawback of the in vitro systems attempting to mimic exocytosis with the reconstituted cell fractions. Indeed, experiments in which membrane fusion between plasma membrane vesicles and chromaffin granules was monitored fluorimetrically revealed only very small degrees of fusion [6]. It is to be noted that other methods for assaying fusion have resulted in considerably higher values [8,9]; however, the sidedness of membrane preparations used in these experiments was not determined. In any case, considerable progress could be expected from the development of techniques for the isolation of plasma membrane vesicles with predominantly inside-out orientation.

We have observed that aggregation of plasma membrane vesicles and chromaffin granules can take place in media with an ionic strength similar to that of the cell cytoplasm, irrespective of whether Mg-ATP was present or absent. Thus, this requirement for exocytosis in 'leaky' cells [2] does not apply for the aggregation observed in the present work. Possibly, charge screening and/or partial destabilization of the structure of one or the other of the subcellular fractions is involved. It is to be noted that aggregation occurred also when the external Ca^{2+} concentration was kept low (approx. 1 μM) by added EGTA. This is distinct from chromaffin granule aggregation, which was reported to require millimolar Ca^{2+} concentrations [34,35] or the presence of a specific aggregation enhancing protein [36,37]. Furthermore, interactions between chromaffin granules provokes clearing of membrane-intercalated particles from the contact site [15,38], while in our experiments the opposite is the case. Our observations suggest that contacts between plasma membrane vesicles (PMV), mostly in right-side-out orientation, and chromaffin granules (CG) entail no clearing of membrane-intercalated particles from contact sites. Only a minor percentage of these PMV-CG aggregates might reflect the situation as it occurs in situ (i.e., inside-out PMV/CG contacts). We had found that, in situ, no membrane-intercalated particles clearing does occur in the cell membrane and chromaffin granule membranes during exocytotic membrane interaction [39]. In the present study a multitude of vesicle contacts in our micrographs are seen at precisely those sites where membrane-intercalated particles are present in clusters, possibly as a consequence of the isolation procedure, involving breakdown of some of the cytoskeletal elements or other structural modifications. Again,

plasma membrane vesicles fractions preferably with inside-out orientation would be of great advantage to mimic membrane interactions in vitro under well defined conditions.

Acknowledgements

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