INTERACTION OF CHROMAFFIN GRANULES WITH PLASMA MEMBRANES MEDIATED BY Ca^{2+} AND Mg^{2+}-ATP USING SELF-GENERATING GRADIENTS OF PERCOLL®

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Received 23 November 1980

1. Introduction

The chromaffin granules (vesicles storing catecholamines, nucleotides and other components of adrenal medullary cells [1] represent a classic model system for exocytotic hormone release from secretory cells [2]. Exocytosis is assumed to involve binding of the storage vesicles to and fusion with, the plasma membrane, and possibly dissociation of (empty) vesicles from exocytotic sites.

Recent evidence suggests that in chromaffin cells at least two components are essential and specific for exocytotic release: Ca^{2+} and Mg-ATP [3]. Here, chromaffin cell homogenates are used to investigate the effect of Ca^{2+} and Mg-ATP on the interaction of granules with fragments of plasma membranes. Although homogenization of tissue certainly destroys cytoplasmatic structures, essential components of the release system remain present.

The main results of this in vitro study [4] are:

(1) Percoll gradients are not only very useful to separate subcellular particles but also to investigate interactions between these particles;
(2) At low Ca^{2+} levels (10^{-7} M) granules and plasma membranes appear to form complexes, independent of the presence of Mg-ATP;
(3) Whereas at higher Ca^{2+} levels (10^{-5}–10^{-4} M) and in the absence of Mg-ATP plasma membranes, granules and lysosomes form (probably unspecific) aggregates, the presence of Mg-ATP (10^{-3} M) causes the separation of granules from complexes with plasma membranes.

Complex formation between granules and plasma membranes at low Ca^{2+} levels and Mg-ATP-mediated dissociation of these complexes at increased Ca^{2+} concentrations is surprising; it has been frequently suggested that in vivo a local increase in Ca^{2+} may cause binding of granules to exocytosis sites of the plasma membranes [5,6] and aggregation between granules [6].

Abbreviations: PIPES, piperazine-*N*,*N*'-bis[2-ethane sulfonic acid]; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*'-tetraacetic acid; (1) buffer (A), 0.3 M sucrose, 20 mM PIPES, 2 mM EGTA, 5 mM magnesium acetate (pH 6.6); (2) buffer (B), as buffer (A) plus 2 mM calcium acetate; (3) buffer (A), 0.75 M sucrose, 50 mM PIPES, 5 mM EGTA, 12.5 mM magnesium acetate (pH 5.85); (4) buffer (B'), as buffer (A') plus 5 mM calcium acetate

2. Materials and methods

2.1. *Homogenate preparation*

In a typical preparation, 5 pairs of bovine adrenal glands (from a local slaughter house) chilled on ice were cut into small pieces after removal of the cortex, divided into 2 equal portions, taken up in buffers A (low Ca^{2+}) or B (high Ca^{2+}), and finely scraped with a scalpel.

Each tissue sample (10 ml buffer/g tissue) was homogenized in a Potter homogenizer with a motor-driven Teflon pestle and centrifuged at 800 × *g*, 10 min. For incubation experiments, 1 ml 0.316 M Na_2-ATP in buffer A (or B), or the same amount of Na_2-ATP as a powder was added to the supernatant. The pH of the incubation medium is 5.9 for buffer A and 6.0 for buffer B. The use of Tris–HCl (pH 7.4) in experiments similar to these without the incubation period leads to comparable results (unpublished). In some experiments low speed centrifugation is carried out after incubation with Mg-ATP (as indicated in figure legends). After 20 min at 37°C in a metabolic shaker samples were diluted with cold buffer A (or B)

to 250 ml final vol. and centrifuged at 26 000 × *g*, 20 min. The latter step separates eventually produced granule ghosts from intact granules as has been found in a control experiment using hypotonically lysed granules. The pellets were resuspended in 5 ml buffer A (or B).

2.2. *Density gradient centrifugation in Percoll®*

The gradient solutions of 60 vol.% Percoll® (Pharmacia) contain 6 ml original Percoll® suspension and 4 ml buffer A′ [3] (or B′ [4]); final pH is 6.6. 0.5 ml Aliquots of resuspended pellets P_2 were mixed with 9 ml Percoll® suspension and each sample was centrifuged at 50 000 × *g*, 30 min, at 2°C (in polycarbonate tubes, Ti 50 rotor, Beckmann-Spinco UC). The pellet material distributed in the self-generated Percoll® gradient was transferred to a fraction collector (LKB) by pumping out with a 2 M sucrose solution at constant speed; the fraction size was 0.4 ml. The fractions were characterized by refractive index measurements (Abbe refractometer); a 20 ml pycnometer was used to calibrate density (g/cm) *vs* refractive index.

2.3. *Enzyme assays*

Acetylcholinesterase (AcChE, EC 3.1.1.7) activity, used as a marker for plasma membranes, was determined according to [7] using a Cary 118 spectrophotometer; β-glucuronidase activity, used as a marker for lysosomes, was determined in each Percoll-fraction diluted 5-fold with H_2O, using a Sigma assay kit [8].

2.4. *Chemical assays*

The content of total catecholamines and nucleotides may be used as markers for chromaffin granules [9]; 0.1 ml aliquots of each fraction were diluted with 5 ml H_2O and sonicated for 15 s (Branson sonifier); then, 0.4 ml 70 vol.% $HClO_4$ was added. The precipitate of Percoll® and protein was removed by (bench) centrifugation and the A_{280} and A_{262} of the supernatant were measured. As a control the trihydroxyindole fluorescence method was applied [10]. Both methods yield the same results.

2.5. *Protein assay in the presence of Percoll®*

Under the conditions of the Bradford protein assay [11], the mixture of a Percoll®-containing sample and the colour reagent slowly becomes turbid. Since the turbidity develops slower than the colour change, a reaction time of 20 s can be used to determine the protein contents with negligible turbidity contribution.

2.6. *Metal ion concentrations*

The concentrations of Ca^{2+} and Mg^{2+} and Ca^{2+}–ATP and Mg^{2+}–ATP complexes were calculated analogous to [12,13]. The pH-dependent apparent stability constants were calculated and then applied for the iteration procedure at a given pH; for the algorithm a program for a Hewlett-Packard HP-95-calculator was written.

3. Results and discussion

It is found that the self-generating Percoll® gradient is a powerful tool, not only for the separation of subcellular particles, but also for the study of interactions between such particles.

As seen from fig.1a, incubation with Mg-ATP at a high Ca^{2+} level (10^{-4} M) leads to a practically com-

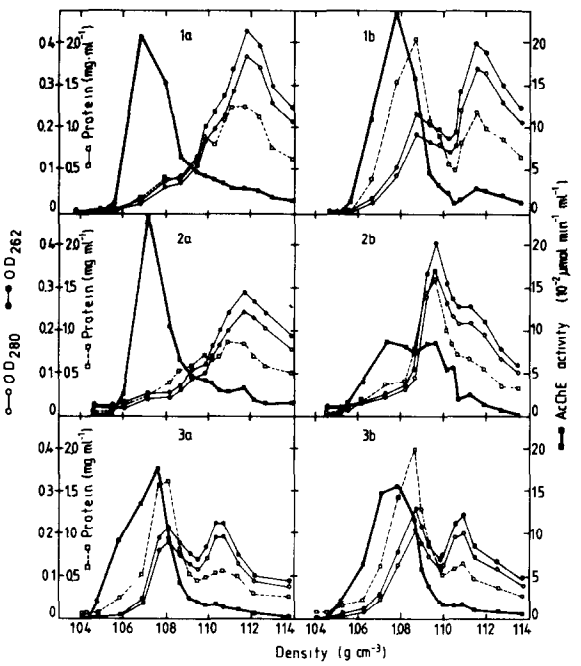

Fig.1–3. Interactions between chromaffin granules and plasma membranes in self-generated Percoll® gradients. The A_{280} (○) and A_{262} (●) indicate the granules; acetylcholinesterase (AcChE) activity (■) marks the plasma membranes: (1) Low speed centrifugation after incubation with 3.2×10^{-3} M Mg-ATP at 37°C in (a) high Ca^{2+} (1.4×10^{-4} M) and (b) low Ca^{2+} (10^{-7} M); (2) Centrifugation before incubation with Mg-ATP and at high Ca^{2+} (1.4×10^{-4} M); (a) incubation with Mg-ATP before and (b) addition of Mg-ATP after dilution with buffer; (3) Samples (a) and (b) as in fig.2, but at low Ca^{2+} (10^{-7} M).

plete separation of granule markers from plasma membrane markers (AcChE-activity). However, at a low Ca^{2+} level (10^{-7} M) a second peak of granule markers appears at an intermediate (Percoll) density; see fig.1b. This peak is associated with higher AcChE activity; the peak of the AcChE activity is shifted to higher density fractions, suggesting complexes between granules and plasma membranes.

As demonstrated in fig.2a,b, the Mg-ATP incubation at high Ca^{2+} levels (10^{-4} M) results in two distinct peaks of AcChE activity. One peak is associated with a major peak of granule markers at intermediate density, and the other one at low density indicates the presence of plasma membranes without granule markers; free granules appear as a shoulder at high density.

A comparison between the results in fig.1,2 indicates that a complete separation between granules and plasma membranes requires both elevated Ca^{2+} levels and incubation with Mg-ATP.

As shown in fig.3, at low Ca^{2+} levels (10^{-7} M) there are aggregates between chromaffin granules and plasma membranes, regardless of whether the samples were incubated with Mg-ATP or not.

In summary, it appears that complexes between granules and plasma membrane fragments are stable at low Ca^{2+} levels (10^{-7} M), irrespective of Mg-ATP incubation. Higher levels of Ca^{2+} (10^{-5}–10^{-4} M) in the absence of Mg-ATP lead to probably non-specific aggregates of granules and plasma membranes, and also of lysosomes (not shown). The presence of Mg-ATP at high Ca^{2+} levels either separates granules from complexes with plasma membranes or prevents the formation of such complexes.

These results are relevant for the preparation of pure plasma membranes and for the analysis of eventual subpopulations of granules. Without precautions the Percoll® density range of the second, low density population of granules found in our study overlaps with that of plasma membranes. For the preparation of pure plasma membranes were therefore suggest to incubate the homogenate in 10^{-4} M Ca^{2+} and Mg-ATP prior to Percoll® density gradient centrifugation. (The 'light granules' found in [14] are possibly aggregates between granules and plasma membranes and identical to our low density population of granules.)

Physiologically, Ca^{2+} and Mg-ATP are essential and specific for exocytotic release of the granular content [3]. These in vitro data suggest that at cytoplasmatic Ca^{2+} levels (10^{-7} M) complexes between granules and plasma membranes are stable and that at higher Ca^{2+} levels (10^{-5}–10^{-4} M) in the presence of Mg-ATP there is a separation of granules from the plasma membranes. Although suggestive, it is at present not clear whether in vitro results represent intermediate states in the initiation and termination of the physiological exocytosis process. To further specify the observed interactions additional marker enzymes have to be used, including those of mitochondria and endoplasmatic reticulum.

Acknowledgements

We thank Mr F. Stocker for excellent technical assistance and the Stiftung Volkswagenwerk for financial support.

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