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ARTEFACTS PRODUCED DURING PLASMA-MEMBRANE ISOLATION

I. CELL DISRUPTION CAUSES ALTERATIONS IN THE STRUCTURE OF THE PLASMA MEMBRANE OF THYMOCYTES

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Summary

Three different methods of cell disruption and plasma membrane isolation without using membrane-stabilising agents, have been compared in order to study whether heterogeneity of the plasma membrane is a natural phenomenon or is induced by cell disruption. The plasma membrane isolated from thymocytes shows an altered structure when compared with the plasma membrane of intact cells. Freeze-etch studies demonstrate an aggregation of intramembranous particles and a decrease in their number. These results are discussed in relation to heterogeneity of the cell surface.

Introduction

In order to carry out structural and functional studies on cell membranes it is first necessary to isolate the plasma membrane as a single entity. Different approaches to this end have been established. One group of techniques stabilizes or partially fixes the cell membrane before cell disruption [1] and the resulting membranes are generally seen as large sheets. However, membrane-bound enzymes are often inactivated by this process and functional studies are therefore impossible. Another group of techniques disrupts the cell without stabilization of the plasma membrane [2] resulting in a membrane fraction consisting of vesicles. This type of isolated membrane should be suitable for functional studies, e.g. stimulation processes produced after interaction with membrane ligand such as mitogens or hormones. Application of data found from

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isolated plasma membranes to the cell membrane of the intact cell requires evidence that the structure of the isolated membrane is the same as that in the unbroken cell. It is, at present, a difficult technical problem in membranology to prepare membranes that resemble the native state.

In several recent publications [3—5] we have proposed the idea that the cell membrane has a heterogeneous structure and is composed of areas differing in chemical composition and biological function. In the case of polarized cells such as hepatocytes and proximal kidney tubuli cells a heterogeneous structure of plasma membrane is obvious and has already been demonstrated, e.g. by Evans [6] and Kinne [7]. We believe that we have established methods [8,9] for separation of such different areas of the plasma membrane of unpolarized cells. It was, therefore necessary to investigate whether this heterogeneity is natural or whether it is induced during cell disruption.

In this paper we show that in thymocytes, cell disruption does in fact induce alterations in the structure of the cell membrane. Plasma-membrane fractions, prepared by three different methods of cell disruption (osmolysis, mechanical disruption and nitrogen cavitation) are compared. All three methods show aggregation of intramembranous particles and reduction of their number.

Methods

Preparation of cell suspensions

Rat (Lewis, 3 months old) or calf (approximately 4 months old) thymus was used. Thymi were removed immediately after death and immersed in phosphate-buffered saline. All further steps were performed at 4°C. Thymi were cut with scissors into small pieces and a cell suspension produced after gentle homogenisation in a loosely-fitting Potter homogeniser. Aggregates and stroma were removed by filtration through 4 layers of cheese-cloth. The thymocyte suspension was washed once with phosphate-buffered saline by centrifugation at 220 $\times g_{av}$ for 7 min and the cell pellet was then resuspended in the appropriate cell disruption buffer. Cell viability after this procedure was between 90 and 97% according to Trypan blue exclusion.

Cell disruption and plasma membrane preparation

Cell disruption by osmolysis and plasma-membrane isolation was based on the method of Cook [10] and is described in detail elsewhere (Bauer et al., in preparation). In brief, $1 \cdot 10^8$—$1 \cdot 10^9$ thymocytes/ml phosphate-buffered saline were added to the osmotic-shocking buffer (20 mM borate, pH 7.2) and magnetically stirred at 500 rev./min for 5 min. The homogenate was filtered through cheese-cloth and then layered over a discontinuous sucrose gradient (42%, and 30%, w/w) followed by centrifugation at 17700 $\times g_{av}$ for 90 min. The upper band was then centrifuged again on a discontinuous sucrose gradient (45%, 35%, w/w) at 177000 $\times$ g_{av} for 90 min. The upper band of this gradient shows typical characteristics of a plasma-membrane fraction.

The mechanical cell disruption was performed using a Stansted cell disrupting pump (Stansted Fluid Power Ltd., Stansted, U.K.) and the plasma membranes were prepared by the method of Crumpton and Snary [11].

The cell disrupting by nitrogen-cavitation was performed as described in

detail by Brunner et al. [9], using a discontinuous sucrose gradient for plasma membrane isolated after differential centrifugation.

Electron microscopic techniques

Small pieces of thymus tissue and thymocytes in suspension were fixed with glutaraldehyde up to 2 h, 2% (v/v) final concentration in phosphate-buffered saline, glycerinated stepwise and frozen on cardboard discs in Freon 22 cooled by liquid nitrogen. Plasma-membrane fractions of the three preparation methods were concentrated by dialysis against polyethyleneglycol, M_r 20000 (30%, w/w) in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer, pH 7.2, or were pelleted by centrifugation at 177000 $\times g_{av}$ for 45 min and resuspended in a small volume of 10 mM HEPES, pH 7.2. These suspension were either fixed with 2% (v/v) glutaraldehyde and glycerinated stepwise or were frozen directly on cardboard discs. Freeze-fracturing and replication was done using a Balzers freeze-etch apparatus at −110°C. The replicas were examined in a Siemens Elmiscope IA or a Philips EM 400, the magnification of which was calibrated with a standard grating replica.

Results

The freeze-fracture electronmicrograph of whole thymus tissue shown in Fig. 1a demonstrates the normal random distribution of the intramembranous particles of the plasma membrane. There is no change in this distribution in intact thymocytes after they have been brought into suspension from thymus tissue (Fig. 1b).

Fig. 1, c—e shows freeze-etch electronmicrographs of the plasma-membrane fractions obtained by the three different isolation techniques. The membrane vesicles can be seen to vary in size depending on the cell disruption method used. Nitrogen cavitation produces membranes vesicles of a diameter between 40 and 140 nm, the maximum of size distribution is at approx. 75 nm.

Mechanical cell disruption results in larger membrane vesicles with diameters between 100 and 340 nm, the maximum of size distribution is approx. 150 nm.

With osmotic cell-disruption the largest membrane vesicles can be obtained, with diameter between 300 and 750 nm, the maximum of size distribution lying at approx. 400 nm.

Fewer membrane particles are seen on the exposed membrane faces from vesicles than in the membrane of the intact cell. All the vesicles obtained from any of the three methods have membrane faces showing either many or only a few particles. The particles are, however, mostly aggregated. It is clear that in contrast to the intact cell there is now a non-homogeneous particle distribution. This is concomitant with the decreased number of particles seen on the exposed membrane faces.

Discussion

Cell disruption is the crucial event in plasma-membrane preparation. In general, not much attention has been paid to this important step in the preparation of plasma membranes and there are as yet no satisfactory methods for well

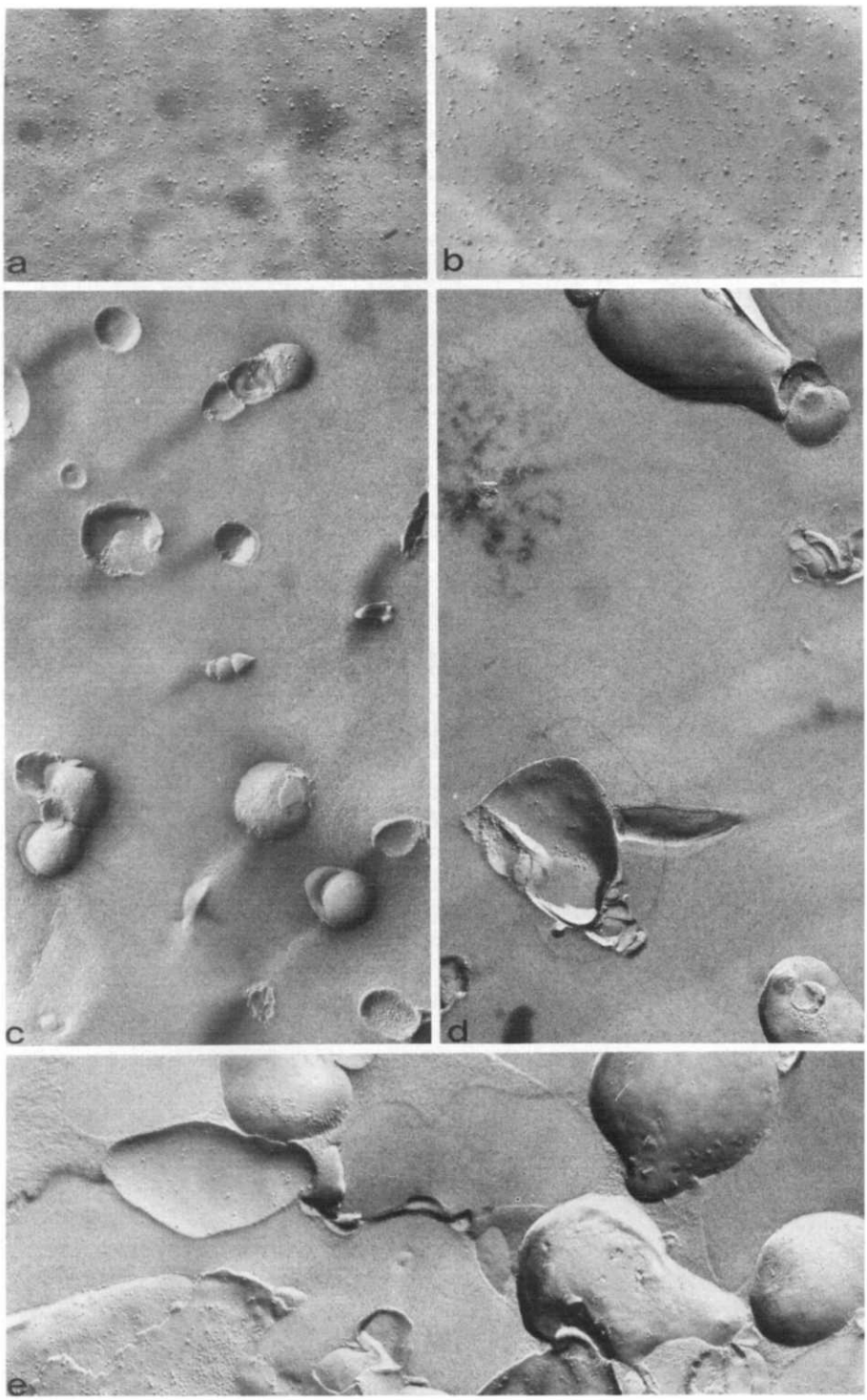

Fig. 1. Freeze-etch aspects of the plasma membrane (P-face) of calf thymocytes. a. Plasma membrane of intact thymocyte in whole thymus tissue. Magnification X 44000. b. Plasma membrane of intact thymocyte in cell suspension. Magnification X 44000. c. Plasma-membrane fraction from thymocytes disrupted by nitrogen cavitation. Magnification X 44000. d. Plasma-membrane fraction from thymocytes disrupted by mechanical forces. Magnification X 44000. e. Plasma-membrane fraction from thymocytes disrupted by osmolysis. Magnification X 44000.

controlled and reproducible cell disruption methods such as nitrogen cavitation also have some disadvantages (Golecki, J. and Brunner, G., in preparation). Few criteria are available that allow a real comparison of the functional abilities of cell membranes, as found in the intact cell, as opposed to the single isolated entity. It is, therefore, difficult to characterize precisely the native state of the cell membrane, or, in other words, to find a measure of the "artificiality" of a plasma-membrane preparation.

This degree of "artificiality" is important for our understanding of regulation processes which take place in the plasma membrane. We suggested that there are two types of plasma membrane-regulated processes; (i) one which involves the membrane alone (theoretically also in the isolated entity), e.g. early membrane events after binding of a membrane ligand (mitogen or hormone), for this process we have proposed the term "first step regulation" [12]; and (ii) a process which requires interaction with cytoplasmic structures.

The comparison here of the number and distribution of intramembranous particles from three differently prepared plasma-membrane fractions (Fig. 1, c, d and e) with that of the whole cell (Fig. 1, a and b) shows that in the case of thymocytes, cell disruption causes, firstly, a remarkable aggregation of their intramembrane particles and, secondly, a decrease in their number.

Particle rearrangement due to osmolysis resulted in areas of about 50—100

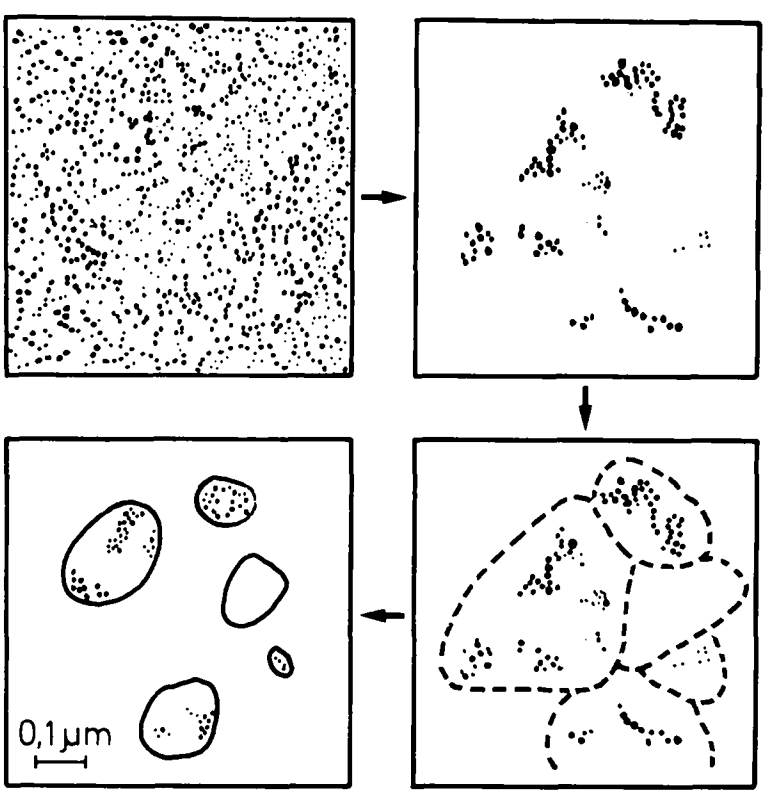

FIg. 2. Possible mechanism for the formation of plasma-membrane vesicles with and without aggregates of intramembranous particles. The last stage shows the size (diameter) of the resulting vesicles.

nm^2 which have either aggregated particles or which are virtually devoid of particles [13]. From these data it can be calculated that heterogeneous vesicle populations are produced, i.e., vesicles possessing large numbers of aggregated particles, vesicles with very few particles and vesicles of mixed type. Fig. 2 depicts our ideas of the formation of the aggregated particles simultaneous with vesicle production. The scheme also gives an impression of the size of the particle aggregates and of the vesicles.

As a second important alteration in the structure of the plasma membrane a decrease in the number of intramembranous particles is induced during cell disruption and plasma-membrane isolation by all three methods used.

These results do not necessarily critizise the concept of the plasma membrane being composed of heterogeneous areas, they merely could show that certain membrane components accumulate preferentially.

In summary it has been demonstrated that cell disruption of thymocytes using methods that do not stabilize the cell membrane, induce changes in the structure of the plasma membrane. This process may exaggerate heterogeneity of the native cell-membrane. It demonstrates clearly that using this approach for plasma-membrane isolation, it is not possible to produce membranes possessing a structural composition similar to that found in the intact cell. This is unlikely to be achieved by methods which do not stabilize the plasma membrane.

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