Biochimica et Biophysica Acta, 649 (1981) 113–120
Elsevier/North-Holland Biomedical Press

BBA 79451

CHANGES OF THE INTERNAL ORGANIZATION OF THE PLASMA MEMBRANE CORRELATED TO THE REGENERATION POTENCY OF THE CELL

V. SPETH [a], H.C. BAUER [b] and G. BRUNNER [c]

[a] *Max-Planck-Institut für Immunbiologie, 7800 Freiburg (F.R.G.),* [b] *Department of Health, Education, and Welfare, National Institutes of Health, Bethesda, MD 20014 (U.S.A.) and* [c] *Institut für Immunologie der Universität, 6500 Mainz (F.R.G.)*

(Received December 12th, 1980)
(Revised manuscript received July 16th, 1981)

Key words: Freeze-fracture; Hypotonic shock; Cytoskeleton; Cell regeneration

Internal organization of the plasma-membrane of rat thymocytes and pituitary tumor cells (GH_3) was experimentally altered by low temperature and either changing osmolarity or adding drugs destroying the cytoskeleton. These treatments induce reversible aggregation of intramembrane particles of the plasma membrane. Thin section electron microscopy of the hypotonically shocked cells show all the cell organelles to be extremely swollen and the cytoplasmic space to be rather empty. Returned to physiological conditions, the cells show normal morphological aspects, accompanied by 'normal' permeability properties of the plasma membrane; the aggregation of intramembrane particles is reversed. The proliferation behaviour of GH_3-cells is not affected by this treatment. This demonstrates a high regeneration potency of the mammalian cell and leads to the assumption that molecular components which are important for the survival of the cell must be structural (membrane) bound.

Introduction

The plasma membrane, the border of the cell, acts as permeability barrier and has a variety of functions as target for receptor molecules [1]. The plasma membrane is fixed to and connected with the cytoskeletal network [2,3] and is able to perform different functions due to a structure of cooperating mobile components [4]. Some of these components are visualised in freeze-etching as intramembrane particles and are assumed to be a part of anchorage proteins of the cytoskeleton [5]. The distribution of these intramembrane particles varies according to the physiological stage of the cell [6]. For the morphological aspect reflecting this stage, which is revealed by freeze-fracturing, the term 'internal organization' of the plasma membrane will be used. For the morphological aspect of labelled receptor molecules on the external surface of the plasma membrane, we introduce the term 'external organization'. In both of these membrane planes one can find aggregation and redistribution phenomena independent of each other. Changes of the external organization can be induced by binding of hormones, antigens and mitogens which induces an aggregation of receptor molecules [7]. Alterations of the internal organization occur during cell-cell interaction-events, such as contact inhibition in monolayers of cells and the formation of junctions.

In this paper, we present data for experimental alterations of internal organization of plasma membranes from two different cell types; isolated thymocytes from rat thymus and cells from a cell culture of a rat pituitary tumor cell line. The goal of the experiments was:

(i) To establish a model for experimental alterations of the internal organization of the plasma membrane as a base for further studies concerning the interaction of intramembrane particles with the cytoskeletal network (i.e. in an aggregated stage and in an evenly distributed stage).

(ii) To correlate alterations of the native structure

of the plasma membrane, e.g. the aggregation and redistribution of intramembrane particles, and the permeability of the membrane as one of the main functions of the plasma membrane.

(iii) To investigate the influence of such molecular events on biological phenomena as cell proliferation to get an impression of the biological importance of such effects.

Materials and Methods

Chemicals. Phosphate-buffered saline, EDTA (ethylenediamine tetraacetic acid) and trypan-blue were obtained from Serva (Heidelberg, F.R.G.). Dulbecco Modified Eagle's Medium was purchased from LS-Laborservice (Munich, F.R.G.); fetal calf serum from Gibco (NUNC, Wiesbaden, F.R.G.), horse serum from Boehringer (Mannheim, F.R.G.) and Trypsin, Neuraminidase, Colchicin and Cytochalasin B from Serva (Heidelberg, F.R.G.).

Preparation and cultivation of cells. Thymocytes were prepared from 6–8 weeks old Lewis rats as described previously [5,8]. GH_3-cells were cultivated at 37°C in Dulbecco Modified Eagle's Medium supplemented with 2.5% fetal calf serum and 15% horse serum in an incubator with 5% CO_2 and 95% humidity in Spinner bottles (Bellco, Tecnomara, Zurich, Switzerland) and culture flasks (75 cm^2 surface, Falcon, LS-Laborservice, Munich (F.R.G.)). For recultivation after different treatments, cells were spread out in cell culture multiplates (Costar, Tecnomara, Zurich, Switzerland). The cell proliferation was measured by counting the cells after desorption from the plate and a single-cell suspension was prepared using Versene. Cells were counted with a heamocytometer.

Hypotonic shock procedure. After harvesting the cells from the spinner culture single-cell suspensions of GH_3-cells were prepared by washing the cells twice in Dulbecco Modified Eagle's Medium without Ca^{2+}. The cells were resuspended in phosphate-buffered saline (310 to 320 mosM) at a concentration of $5 \cdot 10^6$ cells/ml. Using different volumes of double distilled water (brought to the chosen temperature) the different osmolarities were adjusted and the cells were incubated in an ice bath or a water bath at 37°C. The 'isotonization' was restored by adding corresponding amounts of double concentrated buffered saline. The cells were then centrifuged at $700\, g_{av} \cdot$ min and the dead cells counted after addition of trypan blue (final concentration 0.1%).

If trypan blue should be present during the hypotonic treatment, a 0.1% solution in distilled water was prepared. The osmolarity determined by an Osmometer (Knauer, Berlin, Germany) was 20 mosM. 1 ml of the cell suspension was diluted with this solution to the desired osmolarity.

A cytochalasin-B stock solution was prepared by dissolving 1 mg cytochalasin-B in 1 ml dimethylsulfoxide (DMSO). Aliquots of the stock solution were added to cell suspensions to final concentrations of 10–100 μM and incubated for 90–180 min at 37°C. Controls were done by adding DMSO alone to cell suspensions ($2.5 \cdot 10^6$ cells/ml) in the respective concentrations. Colchicine or colcemid was dissolved in phosphate-buffered saline and added to cell suspensions in the desired concentrations.

Electron microscopy. Cells were fixed with 1% glutaraldehyde in 310 mosM phosphate-buffered saline or 65 mosM phosphate-buffered saline at the respective temperature; after 5 min, cells were transferred to an ice bath (4°C) for 30–120 min.

For freeze-etching, cells were glycerinated stepwise up to 25% for 1 h, pelleted and frozen on copper discs in melting propane.

Controls were made by freezing cells in 310 and 65 mosM phosphate-buffered saline directly without fixation or glycerination on copper discs in melting propane.

Freeze etching was performed as described in Ref. 8.

For thin-section electron microscopy, cells were washed in 50 mM cacodylate-buffered after glutaraldehyde and postfixed in 1% OsO_4 in cacodylate buffer at 4°C for 3 h; blockstaining was done in a saturated solution of uranylacetate in 50% ethanol for 1–2 h. After further dehydration in alcohol, the cells were embedded in Epon 812 and cut ultra thin (grey to silver) on a Reichert UMO2 ultramicrotome with a diamond knife. Sections were stained with uranyl acetate and lead citrate.

For ultrastructural viewing a Philips EM400 was used.

Particle counts were performed as described [5] with the aid of a semi-automatic image analyzing system (Leitz ASM; Wetzlar, F.R.G.). For determina-

tion of the coefficient of dispersion (C.D.), the machine was programmed according to Pauli et al. [9,10].

Results

1. Changes of the internal organization

Thymocytes

Normal, freshly isolated thymocytes in suspension show evenly distributed particles on the freeze-fractured plasma-membrane faces. After incubation in hypotonic medium at 110 mosM at low temperature, for 5 min, the particles become reversibly aggregated, particle number is decreased; at 65 mosM, the membrane is already damaged, visible in an irreversible and strong aggregation and diminution of particles. This is in agreement with our previous findings [5].

Aggregation of particles is temperature-dependent; it starts in the temperature-range between 16°C and 8°C and is pronounced below 8°C; aggregation is visible only after 1 min incubation in the shock buffer, and does not become markedly stronger in the interval between 3 and 10 min. (In our experiment, shock was routinely stopped by fixation or re-incubation in physiologic medium after 5 min).

GH_3-cells

The plasma membrane of GH_3-cells in physiologic buffer also reveals normally distributed particles on their fractured E- and P-face (Fig. 1a); particle number is 2972 ± 440 per μm^2 on the P-face, the coefficient of dispersion (as expression for particle aggregation according to Pauli et al. [9,10]) is 0.62 ± 0.18 (Table I), which in a strict mathematical sense means a 'lattice-like' rather than 'random' distribution. This is due to high particle density; with the method used, increasing density of particles automatically leads to 'lattice-like' distributions. This aspect is not changed significantly by temperature or glycerination.

Exposing GH_3-cells to hypotonic buffers, intramembrane particles become aggregated only at 65 mosM and low temperature (Fig. 1b). The coefficient of dispersion is raised to 1.07 ± 0.42, the aggregation is reversible; raising the temperature to above 20°C (normally 37°C) or re-incubation of the cells in 310 mosM above 20°C disaggregates the particle clusters and leads to the original even distribution of intramembrane particles (Fig. 1c, Table I).

The number of particles per square unit is not significantly changed during the shock in GH_3-cells in the range performed (see Table I).

Incubation of cells with trypsin (10 μg/ml, 30 min, 37°C) or neuraminidase (2 μg/ml, 30 min, 37°C) does not significantly affect the freeze-etch aspect of the plasma membrane of GH_3-cells. The subsequent action of one of these enzymes and hypotonic shock does not produce other or more drastic alterations than the use of the hypotonic shock alone.

Cytochalasin B at a concentration of 10–100 μM up to 3 h at 37°C also does not influence the internal organization of thymocytes or GH_3-cells plasma membrane (Fig. 1d). Yet, if the cells are chilled to 4°C after the cytochalasin-B incubation, the plasma membranes reveal drastic aggregation and diminution of intramembrane particles at cytochalasin B concentrations of more than 20 μM (Fig. 1e). This aggregation is reversible by raising the temperature to 37°C. With lower concentrations of the drug the aggregation effects is not distinct; control incubations with DMSO at the respective concentrations (50 μl/ml) do not produce significant particle clustering (Fig. 1f, Table I).

Colchicine or colcemid (10–100 μM), present up to 3 h at 37°C in the cell suspension, do not seem to have pronounced effects on the particle distribution irrespective of fixation temperature (4–37°C). This again is true for thymocytes as well as GH_3-cells.

2. Permeability properties of the plasma membrane

We only present data of GH_3-cells, because recultivation of thymocytes gave rather heterogeneous results and are hard to interprete.

Under the experimental conditions described above, trypan blue enters the cells if present during the hypotonic shock.

Thin section electron microscopy of cells at this stage documents that the changes of the cellular aspect and outline during the hypotonic shock are dramatic: cells fixed after 5 min shock with glutaraldehyde in shock buffer, are rounded, having lost microvilli and other cytoplasmic protrusions; the cellular organelles such as mitochondria, ER, and nuclear membrane are swollen (Fig. 2a). 5 min reincubation of the cells in isotonic medium (phosphate-

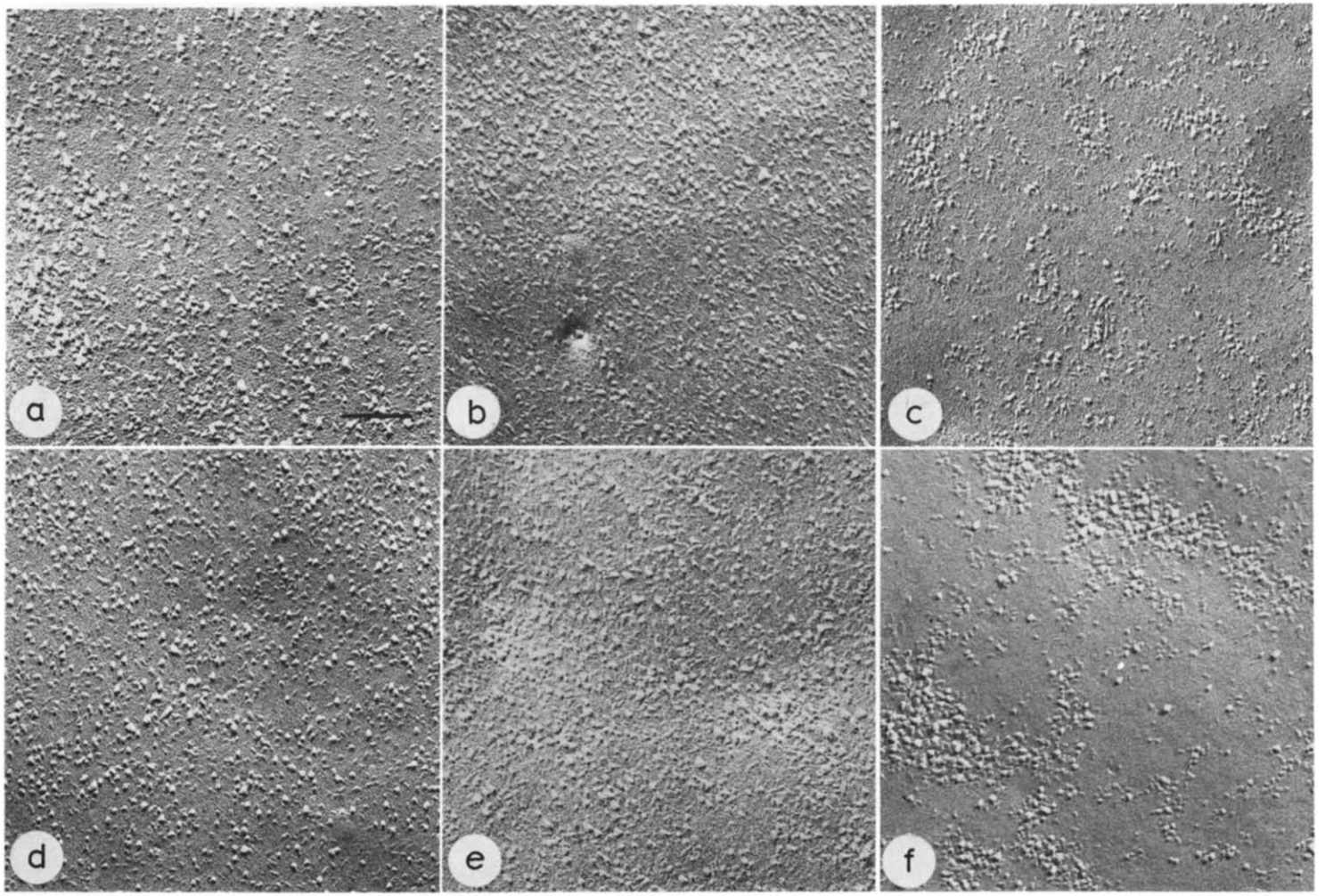

Fig. 1. P-faces of plasma membranes of freeze-fractured GH_3-cells after different pretreatments (cf. also Table I). PBS, phosphate-buffered saline. (a) Control, cells fixed in 310 mosM PBS at 4°C; membrane particles are normally distributed. (b) Cells, after 5 min of hypotonic shock in 65 mosM PBS at 4°C; membrane particles are aggregated. (c) Cells after osmotic shock (65 mosM, 5 min, 4°C) reincubated in 310 mosM PBS for 5 min; membrane particles are again normally distributed. (d) Cells incubated with 20 μM cytochalasin-B at 37°C for 90 min show normally distributed particles. (e) Cells, which are chilled to 4°C after the cytochalasin-treatment reveal aggregated particles in the plasma-membrane. (f) The control-incubation with 50 μl DMSO for 90 min at 37°C and subsequent chilling to 4°C does not change significantly particle distribution. Magnification, all × 100 000, bar represents 0.1 μm.

TABLE I

PARTICLE DENSITIES AND PARTICLE AGGREGATION OF DIFFERENTLY TREATED GH_3-CELLS

PBS, phosphate-buffered saline.

Pretreatment		Number of particles per μm^2 membrane face	Coefficient of dispersion
a	Control, PBS 4°C	2 772 ± 440	0.62 ± 0.18
b	5 min at 65 mosM, 4°C	2584 ± 552	1.07 ± 0.42 [a]
c	5 min at 65 mosM, 4°C then reincubated in PBS, 37°C for 5 min	2 364 ± 460	0.62 ± 0.20
d	20 μM cytochalasin B in PBS at 37°C, 90 min	2 700 ± 420	0.74 ± 0.22
e	20 μM cytochalasin B in PBS at 37°C, 90 min then chilled to 4°C, 5 min	1 768 ± 504 [b]	2.41 ± 0.81 [b]
f	50 μl DMSO in PBS at 37°C, 90 min, then chilled to 4°C 5 min	2560 ± 484	0.83 ± 0.29

[a] Significantly different from a with $P < 0.01$.
[b] Significantly different from d and f with $P < 0.001$.

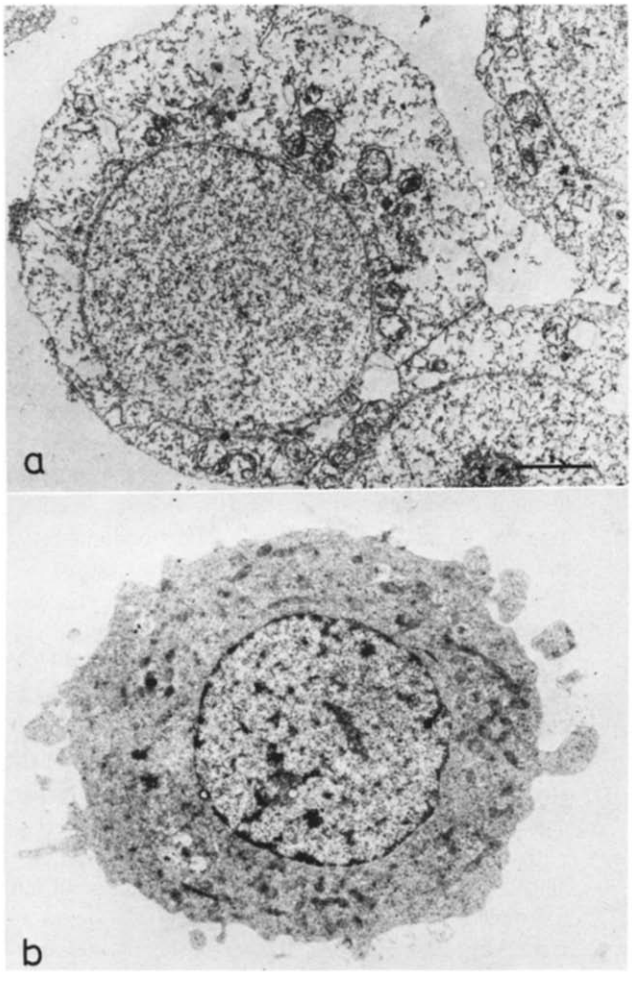

Fig. 2. (a) Thin section of a GH_3-cell, hypotonically shocked for 5 min in 65 mosM phosphate-buffered saline at 4°C (cf. Fig. 1b). The cell is rounded up, cellular organelles are swollen and the cytoplasmic matrix appears rather empty. (b) Reincubation of these shocked cells in 310 mosM for 5 min restores the normal cellular aspects and outline. Magnification both ×5000, bar represents 2 μm.

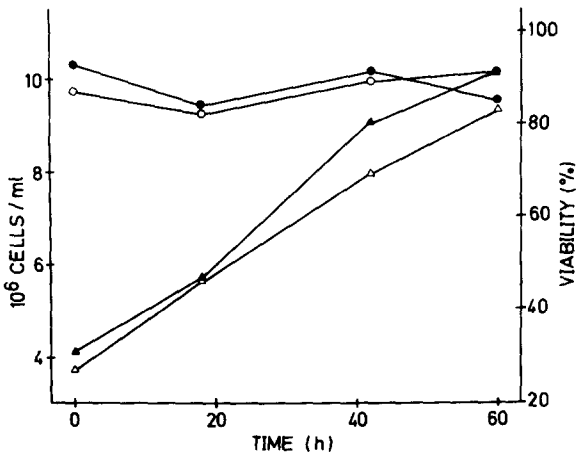

Fig. 3. Viability and proliferation (cell number) of hypotonically shocked GH_3-cells compared to untreated. With respect to shocked cells, time 0 corresponds to state c in Fig. 1 and Table I (5-min reincubation in phosphate-buffered saline after 5 min shock at 4°C and 65 mosM). The figure demonstrates, that GH_3-cells overcome a hypotonic shock at 65 mosM, 4°C for 5 min. Proliferation and viability is not significantly decreased by the shock treatment. ▲———▲, cell number control; △———△, cell number shocked cells; ●———●, viability (living cells in %) control; ○———○, viability shocked cells.

buffered saline) at 37°C leads to nearly complete restoration of the normal cellular aspect, shape and permeability properties (Figs. 2b and 3).

Additionally, control of proliferation during the following 4 days shows that, after 'isotonization' of the medium, the cells survive and proliferate to nearly the same extent as untreated cells (Fig. 3).

Discussion

The results show that the cell can reverse strong disturbances in the plasma membrane and compensate the effect of heavily altered permeability properties.

1. Reversible alteration of the internal organization of the plasma membrane

We demonstrated reversible particle aggregation in the plasma membranes of two different cell types, thymocytes and GH_3-cells, induced at low temperature by hypotonic shock of cytochalasin B.

In thymocytes, the particle aggregation under hypotonic conditions (110 mosM) is accompanied by decreased numbers of intramembrane particles per square unit, as described previously [5].

GH_3-cells are much more resistant to hypotonic solutions. To produce significant particle clustering, this cell type needs shock conditions (65 mosM, 5 min, 4°C) under which thymocytes are already irreversibly damaged [5]. Moreover, in GH_3-cells the particle number is not decreased significantly under shock conditions which lead to particle aggregation.

The reason for the different susceptibility to hypotonic solutions could simply be the difference of cell types, in a way that the plasma membrane of the GH_3-cell per se can be resistant to stronger shocks. However, we do not have direct evidence of a stabilizing effect of e.g. a membrane-surface layer. Digestion with trypsin or neuraminidase did not enhance the particle-aggregating effect of hypotonic shocking in GH_3-cells.

On the other hand, thymocytes, freshly isolated from thymus and brought into single-cell suspension, may already be damaged and stressed by the isolation procedure, and therefore not capable of withstanding further exhausting treatment. An indication for this is the more or less roundish shape of freshly isolated thymocytes, with only a few microvilli and protrusions left (not shown).

In contrast, GH_3-cells exhibit a more irregular outline with protrusions and microvilli. This irregular shape may add or even be the reason for, that the plasma-membrane of this cell type seems to resist stronger hypotonic shocks than the thymocytes. The irregular shape implicates a high surface/volume ratio, and by simply rounding up allows increase of cell volume (influx of water under the hypotonic conditions) without dramatically increasing the cell surface. Furthermore, the number of particles is not reduced during the hypotonic treatment of GH_3-cells. This again indicates, that the plasma-membrane remains largely unstretched and unstressed, under the shock conditions used, though membrane particles are aggregated.

From this, we conclude that particle dislocation is caused by indirect events and is independent of and uncoupled from functional integrity of the membrane: hypotonic shock separates the plasma-membrane from the underlying cytoplasm and thus ruptures cytoskeletal elements, anchored at the plasma-

membrane (intramembrane particles) and extending into the cytoplasm. Whether the plasma-membrane is damaged by this process or not is mainly dependent on the cell shape. In GH_3-cells, plasma-membrane cytoplasm separation is possible without membrane damage because of the irregular (large) surface of the cell; in the case of the rounded thymocytes the plasma-membrane surface has to be enlarged and thus to be stressed, to produce a 'gap' between plasma-membrane and cytoplasm, which ruptures cytoskeletal elements.

The membrane particles, hold and fixed directly or indirectly by these filaments, become mobile upon osmotic shock, and can be aggregated by low-temperature-induced phase-separation phenomenon in the membrane plane.

Looking for the mechanisms involved in the aggregation of intramembrane particles and the nature of filaments, the results with drugs attacking cytoskeletal elements give useful information. In both cell types used in this investigations, we could induce particle aggregation by incubating cells with >20 μM cytochalasin-B for 1–3 h at 37°C and subsequently cooling down the cells to 4°C. Colchicine and colcemid had no effect under the conditions used.

Scott et al. [11] observed a disaggregating effect of cytochalasin-B in naturally aggregated intramembrane particles of 3T3 cells at room temperature. In SV3T3 cells, whose intramembrane particles of the plasma-membrane are normally distributed, no effect of the drug was observed under the tested conditions (incubation up to 8 h at room temperature, 1–500 ng/ml). Also in our experiments, particles are normally distributed at room temperature. Aggregation of intramembrane particles only takes place upon chilling the cells to 4°C.

For the particle aggregation a two-step mechanism is postulated:

(i) Hypotonic shock or cytochalasin-B disrupts actin-like filaments underlying the plasma-membrane; these filaments normally connect directly or indirectly and thus stabilize and immobilize membrane particles.

(ii) Only after disrupting these particle-stabilizing elements, temperature-dependent lipid-protein and lipid-lipid phase-separations, induce reversible clustering of membrane particles.

It is noteworthy that in our experiments, cytochalasin-B-induced particle aggregation is accompanied by reduction of particle number (cf. Table I). This phenomenon is already known from e.g. plasma membranes of thymocytes, where particles are lost and aggregated during isolation procedures [8]; in *Tetrahymena*, particles of the alveolar membranes are reversibly aggregated and diminished upon lowering temperature [12].

As explanation it is conceivable that environmental alterations during demixing of membrane constituents induce conformational changes in the molecules which form intramembrane particles. This may cause dipping out of the fracture plane, or complete dissolving, of membrane particles, depending on what chemical nature the particles are (cf. recent reviews, e.g. Ref. 13).

2. The regeneration potency of the GH_3-cell

During the hypotonic shock, the membrane is permeable to the influx of trypan blue (mol.wt. 956). A slight but significant difference exists in the number of cells stained in dependence of the shock temperature (not shown). An explanation could be that the increased fluidity of the membrane lipids at 37°C makes the membrane elastic and thus more resistant. The cell, after reincubation in isotonic medium can restore to the original state very fast and the membrane shows nearly the same permeability properties as before. The proliferation behaviour of cells after 5-min shock and untreated control cells is the same. If one takes into account the results of our previous findings [5], that also proteins with a molecular weight of 190 000 (lactohydrogenase) can permeate out of the cell then one must assume that in 5 min (shock time) most of the soluble cytoplasmic material is equilibrated with the outer suspension fluid. This means that pools of metabolic substances are diluted up to 500 times. This also corresponds to the electron-microscopic results which show empty cells and swollen organelles during the shock. The considerable loss of material seems to be compensated in a short time. This assumption is based on electron-microscopic results and the proliferation behaviour of the cell. Therefore, one must conclude that most of the components necessary for the survival and the regeneration potency of the mammalian cell should be structural or membrane-bound molecules.

Acknowledgements

The expert technical assistance of Mary Thornton is gratefully acknowledged. Supported by the Deutsche Forschungsgemeinschaft Br 703/4 (G.B.) and SFB 46 (V.S.) Stiftung Volkswagenwerk Az I/35736 (G.B.) and by Max-Planck-Gesellschaft zur Förderung der Wissenschaften (H.C.B.).

References

1 Brunner, G. (1977) Differentiation 8, 123–132
2 Heuser, J.E. and Kirschner, M.W. (1980) J. Cell Biol. 86, 212–234
3 Tsukita, S., Tsukita, S. and Ishikawa, H. (1980) J. Cell Biol. 85, 567–576
4 Cuatrecasas, P. (1974) Annu. Rev. Biochem. 43, 169–214
5 Bauer, H.C., Speth, V. and Brunner, G. (1978) Biochim. Biophys. Acta 507, 408–418
6 Scott, R.E., Carter, R.L. and Kidwell, W.R. (1971) Nature New Biol. 233, 219–220
7 Orci, L., Rufener, C., Malaisse, F., Blondel, B., Amherdt, M., Bataille, D., Freychet, P. and Perrelet, D. (1975) Isr. J. Med. Sci. 11, 639–655
8 Brunner, G., Bauer, H.C., Suter, D. and Speth, V. (1978) Biochim. Biophys. Acta 507, 419–424
9 Pauli, B.U., Friedell, G.H. and Weinstein, R.S. (1978) Lab. Invest. 39, 565–573
10 Weinstein, R.S. (1976) Cancer Res. 36, 2518–2524
11 Scott, R.E., Maercklein, P.B. and Furcht, L.T. (1977) J. Cell Sci. 23, 173–192
12 Speth, V. and Wunderlich, F. (1973) Biochim. Biophys. Acta 291, 621–628
13 Verkleij, A.J. and Ververgaert, P.H.J.T. (1978) Biochim. Biophys. Acta 515, 303–327