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Plasma membrane coating with cationic silica particles and osmotic shock alters the morphology of bovine aortic endothelial cells

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Abstract

We have used a published method of membrane preparation based on the precoating of the apical membrane of aortic endothelial cells with cationic silica microbeads (with or without polyacrylic acid) in combination with an osmotic shock and mechanical shearing to isolate the apical from the basal plasma membranes of these cells, in vitro. After labeling of the plasma membrane of adherent endothelial cells with a fluorescent derivative of phosphatidylcholine and by using laser confocal fluorescence scanning microscopy, we found that this method of membrane isolation rapidly induced invaginations of the basal plasma membrane to an extent which makes this method unsuitable for further membrane lipid analysis. Morphological analysis of the cells and fluorescence recovery after photobleaching experiments on the plasma membranes were performed at each step of the purification procedure and showed that only hypotonic shock and mechanical shearing of the cells enabled the basal plasma membranes to be purified without significant morphological changes.

1. Introduction

Aortic endothelial cells resting on their extracellular matrix occupy a biological interface, in vivo, between the blood and the smooth muscle cells of the arterial wall. The extreme environmental difference between the apical and the basal plasma membrane of this cell monolayer has led to numerous studies related to their respective compositions and functions and then to the recurrent need for efficient methods of plasma membrane isolation. Various approaches can be used for this purpose, among which, the one described by Stolz and Jacobson [1] is particularly attractive. This method is based on a combination of coating of the apical membranes of aortic endothelial cells with cationic colloidal silica microbeads and then polyacrylic acid followed by exposure of the cells to an osmotic shock and mechanical shearing. When using this approach, the basal membrane remains attached to the solid support while the coated apical membranes in suspension are easy to separate from other organelles by centrifugation. However, morphological examination of the preparations at each step of any cell fractionation procedure must be considered as very important. In the case of plasma membranes, this can be done on living cells by the use of laser confocal scanning micros-

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copy. For this purpose, the outer leaflet of the plasma membrane of the endothelial cells was labeled with a fluorescent derivative of phosphatidylcholine [2–4], and the morphology of these non-refringent organelles was checked at each step of their purification. This approach showed that the association of precoating of the cells with cationic silica and exposure to osmotic shock induced dramatic morphological changes in the basal plasma membrane, which render the method unsuitable for further lipid and membrane structure analysis. We show that osmotic shock without precoating of the cells followed by mechanical shearing is preferable to ensure basal plasma membrane isolation. In addition to laser confocal microscopy, the quality of the preparations of basal plasma membranes was assessed by fluorescence recovery after photobleaching (FRAP) experiments.

2. Materials and methods

2.1. Chemicals

1-Acyl-2-[6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]caproyl]-phosphatidylcholine (C6-NBD-PC) was obtained from Avanti Polar Lipids (Pelham, AL, USA). Dioleoylphosphatidylcholine (DOPC) was purchased from Sigma (Saint Louis, MO, USA). Recombinant basic FGF was kindly provided by Dr. Bouche (Institut de Biologie Cellulaire et de Génétique in Toulouse (France)). Cationic colloidal silica microbeads were a generous gift from Drs. B.S. Jacobson and B. Larijani (Lederle Graduate Research Center, Massachussets, USA).

2.2. Cells and cell culture

Vascular endothelial cell cultures were established and cloned twice from ox aortic arch and characterized as previously described [5–7]. In most experiments, cells resting on 22×22 mm coverslips were used near confluence because membrane isolation procedure occasionally resulted in whole detachment of postconfluent endothelial cell monolayers as an intact sheet or in poor basal plasma membrane recovery as previously reported by Stolz and Jacobson [1].

2.3. Isolation of plasma membrane domains from tissue culture coverslips

The procedure used for the coating of the cells was that described by Stolz and Jacobson [1]. Endothelial cell monolayers were washed twice with cold PBS and then twice with the coating buffer, CB (20 mM 2[*N*-morpholino]ethanesulfonic acid, 130 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 6.5). Cells were coated for 10 s with a 5% (w/v) cationic colloidal silica suspension in CB which was subsequently diluted 10-fold by the addition of CB in the Petri dishes. The monolayers were rinsed four times with CB and exposed (or not) for 10 s to a solution of polyacrylic acid (1 mg/ml in CB) which was then diluted 10-fold with CB. After one wash with lysis buffer, LB (2.5 mM Tris, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.4), the cells were allowed to stand on ice for 3 min in LB. To isolate their basal plasma membranes, mechanical shearing was applied to the cell monolayer by squirting the cells with cold LB at about a 45° angle using a 5-ml syringe fitted with a flattened 18-gauge needle. The progress of cell lysis was continuously monitored under a phase-contrast microscope. Isolated basal plasma membranes anchored on glass coverslips were finally rinsed three times with PBS.

2.4. Plasma membrane labeling and fluorescence intensity measurements on endothelial cells

All these procedures were performed as described previously [2–4]. Mixed vesicles of DOPC and the fluorescent probe C6-NBD-PC, in the 1/1 (w/w) ratio were prepared by injection of an ethanol solution of the lipids in PBS at room temperature [8]. The final lipid concentration was 40 μg/ml (20 μg/ml of each lipid) and the vesicles were used within 1 day. Endothelial cell monolayers were washed twice with cold PBS and chilled on an ice-water bath for 10 min. The medium was removed and cells were incubated for 10 min (still on an ice-water bath) with a cold lipid vesicle suspension containing C6-NBD-PC. Fluorescence originating from the membrane surface was measured with the FRAP apparatus, by illuminating small basal plasma membrane areas of 1.95 μm radius either on entire cells or on isolated sheets. For each measurement, 120–cells were examined.

2.5. Fluorescence recovery after photobleaching experiments

FRAP experiments were carried out under conditions of constant incident light intensity and of 'uniform disk illumination' using apparatus and experimental conditions previously described [9]. The diffusion coefficient D and the mobile fraction M were obtained by double fitting of the experimental recovery data, using a finite differentiation method and statistical analysis [9]. D and M values are given at 95% confidence level. FRAP experiments were carried out at a temperature of 20°C and at least 60–80 cells were tested for each kind of experiment.

2.6. Confocal fluorescence microscopy

Cells were observed with the LSM 410 inverted confocal microscope from Zeiss (Germany) equipped with a ×63 oil-immersion objective. Excitation and emission wavelength were 488 and 530 nm, respectively.

3. Results

3.1. Morphological analysis

When compared to control cells (Fig. 1A), coating of endothelial cells with colloidal silica and polyacrylic acid did not produce obvious morphological change (Fig. 1B) and a homogeneous labeling pattern of the plasma membrane could be seen in both cases. A subsequent brief exposure (less than 3 min at 4°C) to hypotonic buffer (Tris 2.5 mM, 0.5 mM Ca^{2+}, 1 mM Mg^{2+}, pH 7.2) brought about a large swelling of the cells with the appearance of intracellular fluorescent labeled vesicles located at the cell junctions or originating from the basolateral domain (Fig. 1C). After 5–10 min of this treatment, fluorescence appeared mainly in grain-like forms inside the cells (data not shown). In contrast, exposure of the cells to the hypotonic buffer only led to an instantaneous and large expansion of the apical plasma membrane, but without appearance of the vesicles (Fig. 1D). Because the folding of the plasma membrane at junctional sites could provide sufficient material, when stretched, to explain the origin of the large apical expansion, only osmotic shock was performed on sparse cells. Homogeneous membrane labeling was observed in swollen sparse cells and an instantaneous and large apical membrane expansion could be observed (Fig. 1E) while their basal area remained nearly unchanged when compared to control cells (data not shown). In the swollen near-confluent and sparse cells, fluorescence appeared to be less intense in the apical than in the basal pole (see Z sectioning in Fig. 1D,E). This could be due to the fact that the probe was relatively more diluted in the apical than in the basal pole and/or to attenuation of the recorded fluorescence intensities with increasing penetration of the laser beam in the watery medium. For that reason and to determine more precisely the origin of this membrane expansion, the fluorescence intensities originating from the basal membrane were compared after each of the following conditions: (1) confluent cells labeled with C6-NBD-PC; (2) cells labeled and then exposed 3 min at 4°C to the hypotonic buffer; and (3) cells first exposed to the hypotonic buffer and then labeled. The means of the fluorescence intensities per unit of membrane area were found to be equal when the labeling was performed on native plasma membranes or when the labeling was performed after the osmotic shock on the swollen cells. However, we have observed that when the cells were first labeled and then exposed to the hypotonic medium, the swelling of the labeled cells is accompanied by a decrease of about 40% of the fluorescence intensities per unit of membrane area. This suggests that the membrane surface area which could be labeled with the fluorescent probe after the osmotic shock originated probably from a pre-existing intracellular compartment. However, when the swollen cells were disrupted by squirting the cell monolayer with the hypotonic buffer, the resulting basal plasma membrane displayed homogeneous labeling whenever the labeling step was performed, i.e. on the native cells before the isolation procedure (Fig. 1F), after the osmotic shock on unlabeled cells, or directly on the purified basal plasma membrane (data not shown).

3.2. Fluorescence recovery after photobleaching experiments

To assess the structural integrity of the basal mem-

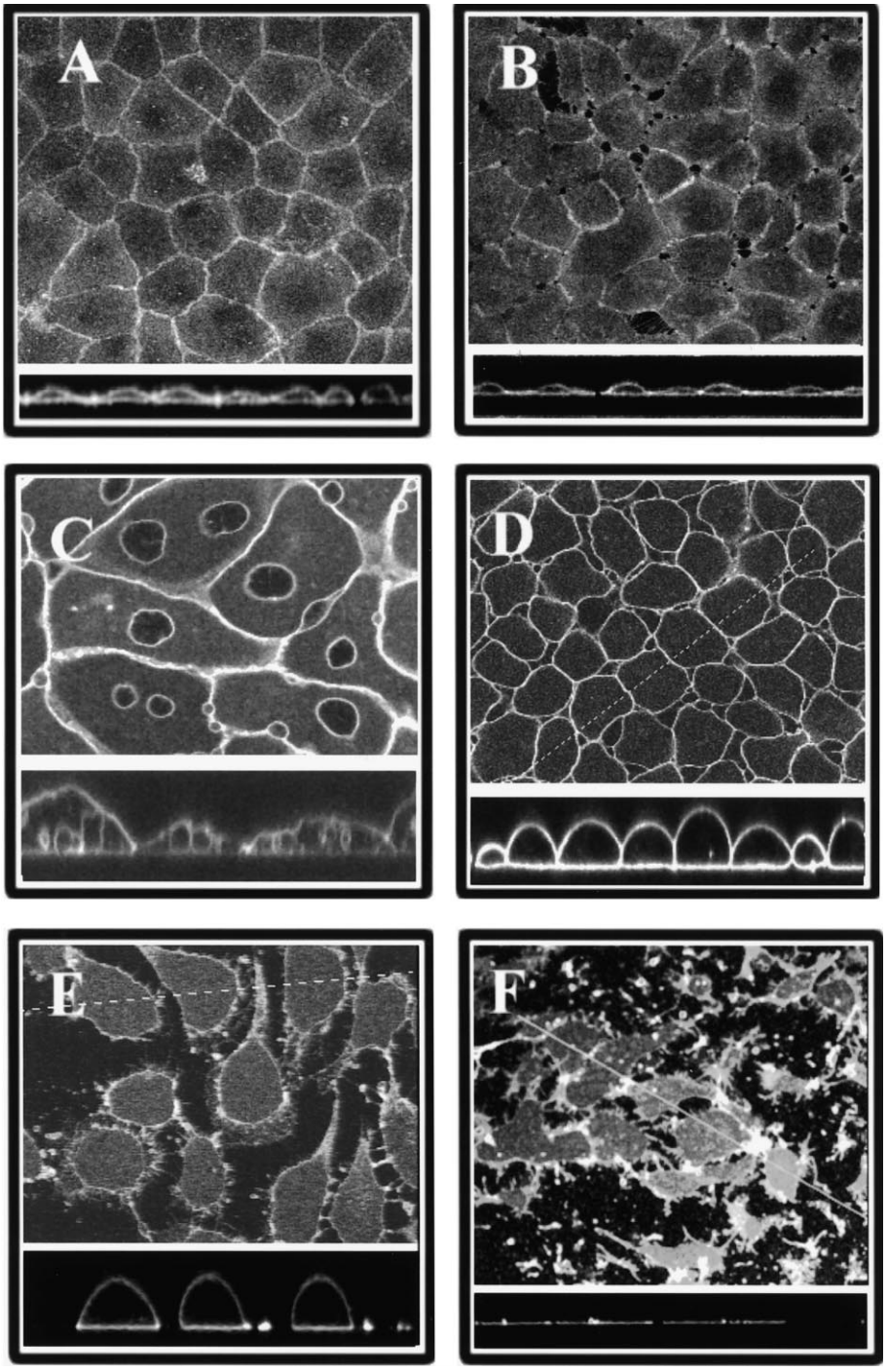

Fig. 1. Laser confocal fluorescence scanning microscopy of vascular endothelial cells labeled with C6-NBD-PC. (A) Micrograph of confluent cultures before and (B) after coating with cationic silica or (C) after coating with cationic silica and exposed to hypotonic shock or (D) exposed only to the lysis buffer. In E, a micrograph of labeled sparse cells incubated in the hypotonic medium is shown; and in F, basal plasma membranes isolated from confluent cells after osmotic shock and mechanical shearing. The upper part of each micrograph was obtained in the *xy* plane while the lower part is an optical Z sectioning of the corresponding sample following the white line when indicated. The entire width of micrographs was 200 μm in A, B, D and F, and 80 μm in C and E.

brane obtained from swollen cells, FRAP experiments were carried out on the basal pole of either whole cells before or after swelling and on isolated basal membrane domains. The lateral diffusion coefficient of C6-NBD-PC was found to slightly increase in the basal pole of entire cells labeled before or after the osmotic shock ($D = 1.8 \pm 0.2 \times 10^{-9}$ cm^2/s) when compared to control cells ($D = 1.1 \pm 0.1 \times 10^{-9}$ cm^2/s, Table 1). Concomitantly, the fraction of probes which were free to diffuse decreased from $87 \pm 3\%$ in control cells to $75 \pm 3\%$ in swollen cells (Table 1). Lateral diffusion parameters obtained in basal membranes after their isolation ($D = 6.7 \pm 0.3 \times 10^{-9}$ cm^2/s and $M = 81 \pm 3\%$, Table 1) indicated either a further modification of membrane properties after disruption of the cells or a possible redistribution of the fluorescent probe between the two leaflets of the plasma membrane or both. However, it should be noted that the fraction of the probes which were free to diffuse in isolated basal plasma membranes ($M = 81\%$) was similar to that measured on whole cells ($M = 87\%$). Large M value is a good indication of the absence of contamination by intracellular organelles. In fact, they would contribute to the photobleaching process, but their small sizes could not constitute surfaces sufficiently large to allow the recovery process in the area of the measurements. In this respect, FRAP experiments performed on basolateral domains obtained after mechanical shearing of silica-coated and swollen cells produced non-measurable D and M values. Very low M values were also recorded on unsuccessful preparations which, in this case, were due to intracellular membranes. This indicates a disruption of the lateral diffusional continuum of the lipids which may be correlated to the appearance of the intracellular vesicles described above (see Fig. 1C) when the cells are precoated with colloidal silica beads.

Table 1
Diffusion coefficient D and mobile fraction M of the fluorescent derivative of phosphatidylcholine inserted in the plasma membrane of bovine aortic endothelial cells exposed or not to hypotonic medium, or after basal plasma membrane isolation without cationic silica precoating.

	D (10^{-9} cm^2/s)	M (%)
Cells labeled in isotonic medium	1.1 ± 0.1	87 ± 3
Cells labeled in isotonic medium and exposed to hypotonic medium	2.0 ± 0.1	76 ± 2
Cells labeled in hypotonic medium	1.8 ± 0.2	75 ± 3
Isolated basal plasma membranes	6.7 ± 0.3	81 ± 3

4. Discussion

Membrane isolation procedure using precoating of numerous cell types with cationic silica particles [1,10–14] proved to be a very useful tool in the course of the identification of the proteins associated with the plasma membrane compartment, as well as in the purification of associated structures like caveolae [15,16]. However, little attention has been focused on the behavior of their lipid molecules in the course of the purification process, probably owing to severe technical difficulties locating these ubiquitous molecules precisely. In the present study, we wanted to use this efficient method to further determine the lipid composition of the apical vs. the basal plasma membrane of aortic endothelial cells and a major aim was, of course, to avoid the contamination of the isolated fractions with intracellular lipids. The data presented above clearly indicate dramatic plasma membrane remodeling when using the combination of coating the apical membrane with cationic silica (with or without polyacrylic acid) and osmotic shock. For that reason, only osmotic shock and mechanical shearing were further used. However, with the exception of bovine corneal endothelial cells which disrupt instantaneously in distilled water ([17] and unpublished observations), the swelling of mammalian cells under hypotonic conditions is a well known phenomenon but little attention has been focused on the origin of the new emerging plasma membrane material. Here we show that this apical plasma membrane surface could not be accounted for by the folding of the plasma membrane at the cell junctions nor by the stretching of microvilli which are not present in the aortic endothelial cells [7]. However, as observed on giant unilamellar vesicles, contribution of stress induced apparent area changes under hypotonic conditions cannot be excluded [18]. The origin of the intracellular lipids mobilized in the plasma membrane during the osmotic shock remained to be determined. Lastly, the phosphatidylcholine fluorescent analog inserted in the purified sheets of basal plasma membrane domains exhibited a higher M value than

in entire cells. This could be related to a global change in membrane structure, but also to randomization of lipids between the two leaflets of the plasma membrane. However, such complete randomization was already observed for a series of partially water soluble phospholipid probes during hypotonic hemolysis of human erythrocytes in the absence of Mg-ATP, i.e. in the absence of aminophospholipid translocase activity [19]. In view of the previous work of Stolz et al. [1] and of the present study, it seems reasonable to propose that in association with mechanical shearing, the use of the precoating of cells with cationic silica and an osmotic shock is suitable only for the isolation of the apical membrane of the aortic endothelial cells, but an osmotic shock without coating is preferable for isolation of the basal ones.

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