THE DISTRIBUTION AND MOBILITY OF ANIONIC SITES ON THE SURFACES OF BABY HAMSTER KIDNEY CELLS

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ABSTRACT

The distribution and mobility of anionic sites on the surfaces of baby hamster kidney cells were studied by utilizing the multivalent ligand, polycationic ferritin, as a visual probe. Our observations revealed that anionic sites are distributed over the entire cell surface, with the highest density of sites being located on cell surface microextensions. Following the initial binding of polycationic ferritin to the surface of unfixed cells, the ligand-bound anionic sites redistributed by migrating from the surface of microextensions to the surface of the cell body. In 20 min, this migration resulted in a total clearing of anionic sites from the surface of microextensions concomitant with the formation of patches of anionic sites on the surface of the cell body. Polycationic ferritin-induced migration and patch formation of anionic sites was not prevented by 2,4-dinitrophenol, N-ethylmaleimide, colchicine, or cytochalasin B. However, the ligand-induced redistribution of cell surface anionic sites was prevented by prefixation of cells with glutaraldehyde.

Various studies have revealed that all vertebrate cell surfaces contain a net negative charge (20). More than 90% of the total cell surface charges are anionic (14). Such charged sites on the cell surface consist of the carboxyl groups of neuraminic acids of glycoproteins and glycolipids (14, 20), the carboxyl groups of aspartic and glutamic acids of proteins and glycoproteins (14, 20), the carboxyl and sulfate groups of mucopolysaccharides (13), and the alkaline phosphatase- and ribonuclease-sensitive phosphate groups which may be associated with cell surface ribonucleic acid (14, 20).

We were interested in studying the distribution of anionic sites on the surfaces of baby hamster kidney (BHK) cells and determining whether such sites would undergo ligand-induced redistribution in the lateral plane of the plasma membrane. Such redistribution has been reported to result from the binding of bi- or multivalent ligands, such as immunoglobulin (2) or concanavalin A (17), to appropriate cell surface receptor sites, and is consistent with the fluid mosaic model of the plasma membrane (5, 18).

The distribution of anionic sites on cell surfaces has been studied by the colloidal iron technique (6). This technique is carried out at low, nonphysiological pH and requires the use of fixed material. Recently, Danon et al. (2) have described an alternative technique for visually probing the anionic sites of cell surfaces by using polycationic ferritin, a multivalent ligand which can be bound at physiological pH to either fixed or unfixed cell surfaces. Presumably, this multivalent ligand can bind to and cross-link most, if not all, membrane macromolecules containing anionic groups exposed at the cell surface.
We report here our studies on the distribution and mobility of anionic sites on the surface of BHK cells, utilizing polycationic ferritin as the visual probe. These studies were previously reported in abstract form (9).

MATERIALS AND METHODS

BHK-21-13s cells were grown in suspension culture in "Wistar" medium. The culture medium was Eagle's minimal essential medium (MEM) (spinner modified) with double the concentration of amino acids (except 1× glutamine) and vitamins and supplemented with 0.1 g/liter ferric nitrate, 2.0 g/liter d-glucose, 10% tryptose phosphate broth, and 10% fetal calf serum. The final sodium bicarbonate concentration in the medium was 0.5 g/liter.

Cells were labeled with polycationized ferritin as follows. Suspension culture cells in the logaritmic growth phase were collected by centrifugation at 500 × g for 2 min and resuspended in Dulbecco's phosphate-buffered saline (PBS) at a concentration of 2 × 10^6 cells/ml. Polycationic ferritin (8 mg/ml) was added to 1/2 ml aliquots of the cells at the desired concentration, and the incubations were carried out at room temperature for time periods varying from 10 s to 30 min. The reactions were stopped by the addition of 10 vol of PBS to the incubations and then a solution of 2% glutaraldehyde in PBS was added to the cells to attain a final glutaraldehyde concentration of 0.2%. The cells were pelleted by centrifugation at 500 × g for 2 min and prepared for transmission electron microscopy by thin-section techniques as described below. In control experiments, in which native ferritin was used in place of polycationized ferritin, there was no nonspecific binding of the ligand to the cells.

In experiments requiring prefixation of the cells, a solution of 2% glutaraldehyde in PBS was added to 2 × 10^6 cells/ml in PBS to attain a final glutaraldehyde concentration of 0.2%. The cells were incubated for 10 min at room temperature, at which time a solution of 0.1 M NH₄Cl in PBS was added to attain a final NH₄Cl concentration of 0.01 M. 10 vol of PBS were added, and the cells were pelleted by centrifugation at 500 × g for 2 min. These prefixed cells were then resuspended in PBS at a concentration of 2 × 10^6 cells/ml and used for experimental purposes as described above. The addition of NH₄Cl was necessary to preclude glutaraldehyde cross-linking of the cells during centrifugation which would prevent their subsequent resuspension.

In the cells to be processed for thin-sectioning, pellets prepared as outlined above were resuspended in 3% glutaraldehyde in 0.1 M Na phosphate buffer (pH 7.4) at room temperature. Aliquots were centrifuged into micro-centrifuge tubes and fixed for 2 h at 4°C. The micro-centrifuge tubes were washed with 0.2 M sucrose in 0.1 M Na phosphate (pH 7.4) and postfixed in 2% osmium tetroxide in 0.1 M Na phosphate buffer (pH 7.4) for 1 h. Gray to silver sections of Epon-embedded micropellets were stained for 5 min in saturated uranyl acetate in 50% ethanol followed by 4 min in lead hydroxide.

Electron micrographs were taken on a JEOL 100B electron microscope at 60 kV with a 40 μm objective aperture.

BHK cells which were suspension culture-adapted (BHK-21-13s) were the gift of Dr. Adrian Chappel, Communicable Disease Center, Atlanta, Ga. Eagle's MEM (spinner modified), MEM amino acids, MEM vitamins, and fetal calf serum were obtained from Grand Island Biological Co., Grand Island, N. Y. Colchicin and cytochalasin B were purchased from Calbiochem, San Diego, Calif. 2,4-dinitrophenol, N-ethylmaleimide, and N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) buffer were purchased from Sigma Chemical Co., St. Louis, Mo. Native ferritin and polycationic ferritin were obtained from Miles Laboratories, Kankakee, Ill. Other reagent grade chemicals were purchased from Fisher Scientific Co., Houston, Tex.

RESULTS

Redistribution of Anionic Sites on the Surfaces of Unfixed Cells

After very brief (10 s) treatment of BHK cells with a high concentration of polycationic ferritin (0.32 mg/ml) the location of the bound ligand on the cell surface revealed that anionic sites were randomly distributed on the plasma membrane including the cell microextensions and cell body (Fig. 1). The anionic sites appeared to be arranged as single sites as well as small clusters of sites. After 1 min incubation in the presence of polycationic ferritin, a striking redistribution of the anionic sites became apparent. Fewer sites were located on the microextensions while small patches of sites were apparent on the cell body (Fig. 2). In distinct contrast, we found that the distribution of anionic sites on prefixed cells was completely random following a 1 min incubation with polycationic ferritin (Fig. 3).

Further incubation of unfixed cells for 5 min (Fig. 4) and 20 min (Fig. 5) with polycationic ferritin revealed a greater change in the distribution of the cell surface anionic sites. It is especially noteworthy that the bound ligand induced a rapid disappearance of anionic sites from cell surface microextensions, while larger patches developed on the cell body. Patches of polycationic ferritin often persisted between the plasma membranes of the microextensions and cell body, presumably linking the two regions electrostatically (Fig. 5). Pinocytosis, resulting in the uptake of polycationic...
FIGURE 1 BHK cell incubated 10 s with 0.32 mg/ml polycationic ferritin before cell fixation. The ligand is randomly bound to anionic sites on cell microextensions and the cell body. × 51,000.
FIGURE 2 BHK cell incubated 1 min with 0.32 mg/ml polycationic ferritin before cell fixation. The ligand is diminished on the ends of microextensions while small patches of ligand are apparent at the base of microextensions and on the cell body. Local pinocytosis can be observed. x 51,000.

FIGURE 3 BHK cell incubated 1 min with 0.32 mg/ml polycationic ferritin after cell fixation. The ligand is distributed randomly and diffusely over the entire surface of the cell microextensions and the cell body. Pinocytosis is not observed. x 51,000.
**Figure 4** BHK cell incubated 5 min with 0.32 mg/ml polycationic ferritin before cell fixation. The ligand has nearly cleared from the cell microextensions while patch formation is obvious on the cell body. × 51,000.

**Figure 5** BHK cell incubated 20 min with 0.32 mg/ml polycationic ferritin before cell fixation. Microextensions are essentially free of the ligand. Large patches of ligand persist on the cell body as well as between the cell body and microextensions which have bent over. A number of pinosomes are obvious. × 51,000.
FIGURE 6 BHK cell incubated 10 s with 0.08 mg/ml polycationic ferritin before cell fixation. The ligand is bound to high density anionic sites which are restricted to the surface of microextensions. × 51,000.

Localization and Redistribution of High Density Anionic Sites on the Cell Surface

In the experiments described above, a high concentration of polycationic ferritin was employed to extensively label the cell surface. Treatment of cells with a lower concentration of polycationic ferritin (0.08 mg/ml) for 10 s revealed preferential localization of small clusters of anionic sites on cell microextensions (Fig. 6). After treatment of cells with this concentration of polycationic ferritin for 1 min, the anionic sites diminished on the microextensions and appeared in patches on the cell body (Fig. 7). When cells were prefixed with glutaraldehyde and subsequently treated with polycationic ferritin for 10 s, binding was preferentially localized on cell surface microextensions as found with unfixed cells (Fig. 6). However, incubation of prefixed cells for 1 min with 0.08 mg/ml polycationic ferritin revealed a random distribution of anionic sites on the surfaces of both the cell body and microextensions (Fig. 8). These findings revealed that the binding of polycationic ferritin depends upon both concentration...
FIGURE 7  BHK cell incubated 1 min with 0.08 mg/ml polycationic ferritin before cell fixation. The bound ligand is diminished on the cell microextensions and appears as patches on the surface of the cell body. × 51,000.

FIGURE 8  BHK cell incubated 1 min with 0.08 mg/ml polycationic ferritin after cell fixation. The ligand is randomly distributed over the entire surface of the cell. × 51,000.
The Effect of Various Conditions on Redistribution of Anionic Sites on the Surfaces of BHK Cells*

| Treatment | Glutaraldehyde fixation | Polycationic ferritin time | Redistribution |
|-----------|-------------------------|---------------------------|----------------|
|           |                         | mg/ml | min |                 |
| Experiment 1 |                       |       |     |                 |
| No additions | Postfixed | 0.32 | 20  | Yes             |
| No additions | Prefixed  | 0.32 | 20  | No              |
| Colchicine, 5 x 10^-4 M, 5 min | Postfixed | 0.32 | 20  | Yes             |
| Cytochalasin B, 80 μg/ml, 5 min | Postfixed | 0.32 | 20  | Yes             |
| N-ethylmaleimide, 10^-4 M, 5 min | Postfixed | 0.32 | 20  | Yes             |
| Experiment 2 |                       |       |     |                 |
| No additions | Postfixed | 0.08 | 30  | Yes             |
| No additions | Prefixed  | 0.08 | 30  | No              |
| Colchicine, 5 x 10^-4 M, 10 min | Postfixed | 0.08 | 30  | Yes             |
| Cytochalasin B, 80 μg/ml, 10 min | Postfixed | 0.08 | 30  | Yes             |
| Experiment 3 |                       |       |     |                 |
| No additions | Postfixed | 0.32 | 5   | Yes             |
| No additions | Prefixed  | 0.32 | 5   | No              |
| 2,4-dinitrophenol, 10^-4 M, 10 min | Postfixed | 0.32 | 5   | Yes             |
| N-ethylmaleimide, 10^-4 M, 10 min | Postfixed | 0.32 | 5   | Yes             |

* Cells were subjected to treatments as indicated and then the polycationic ferritin was added to the incubations for the time periods shown. Fixation was carried out before (prefixed) or after (postfixed) polycationic ferritin treatment. Other details are described in the Materials and Methods section.

DISCUSSION

The major findings presented in this communication are as follows. (a) Anionic sites were located randomly over the surface of BHK cells; however, anionic sites which showed the highest affinity for polycationic ferritin were located on cell surface microextensions. (b) Binding of surface anionic sites by polycationic ferritin induced a directional flow of sites from cell surface microextensions onto the cell body. (c) After a 20 min incubation of cells with polycationic ferritin, anionic sites were found to be arranged in dispersed patches on the cell body, bound between the surfaces of the cell.
that polycationic ferritin has been used previously as a visual probe to identify the normal distribution of anionic sites on erythrocytes (2) and to verify the redistribution of surface anionic sites and intramembrane particles of erythrocyte ghost membranes which were induced to patch by lowering of the pH (16). It was not previously reported, however, that the binding of polycationic ferritin to cell membranes per se resulted in redistribution of surface anionic sites.

It is important to note that progressive reorganization of anionic sites on the BHK cell surface was not prevented by inhibitors of cell metabolism (2,4-dinitrophenol and N-ethylmaleimide) or by inhibitors of microfilament (cytochalasin B)- or microtubule (colchicine)-mediated processes. Thus, the redistribution of cell surface anionic sites described here is another example of ligand-induced patching of plasma membrane components that does not require metabolic energy. It should be mentioned that energy-requiring cap formation was not observed in the present study; however, incubation with polycationic ferritin beyond 30 min was not investigated in these inhibition studies.

Various functions have been attributed to anionic sites on the cell surface. It has been suggested that the initial event in pinocytosis or phagocytosis is the binding of cationic substances to the cell surface (1, 15) and that change in cell surface charge leads to alteration in membrane permeability and transmembrane potential (1, 15) as well as to change in cell movement and chemotaxis (12, 22). Our observation that the highest density of anionic sites is associated with cell surface micro-extensions is especially significant since cell surface extensions may be the initial sites of cell shedding of surface components that has been casually described by many investigators studying cell shedding of surface components that has been casually described by many investigators studying cell surface movements with visual techniques. Chemical studies have shown that immunoglobulin is shed from the surface of B lymphocytes (19) while cell surface receptors for wheat germ agglutinin and H2 antigen have been reported to shed from chick embryo cells (11). These investigators demonstrated a chemical similarity between shed
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