Structural Changes in BHK Cell Plasma Membrane Caused by the Binding of Vesicular Stomatitis Virus

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Spin label electron spin resonance techniques using a nitroxide derivative of stearic acid were used to detect changes in plasma membrane structure caused by the binding of vesicular stomatitis virus (VSV) to cell plasma membranes of intact BHK-21 cells. The results indicate that binding of VSV to cell surface receptors causes an increase in the observed rigidity of the plasma membrane lipid bilayer. This change in membrane structure, which appears to be caused by the cross-linking of receptors in the plane of the plasma membrane, could be prevented by treating the cells with colchicine before addition of virus and could be reversed by treating the cells with colchicine after addition of virus. Cells treated with a monovalent, water-soluble derivative of VSV G-protein (G_s) did not show an increase in plasma membrane bilayer rigidity. However, addition of anti-VSV G-protein immunoglobulin G to cells pretreated with G_s caused an increase in plasma membrane bilayer rigidity. This increased rigidity could also be reversed by the addition of colchicine. Fluorescence microscopy was used to determine the distribution of fluorescein-labeled VSV particles on the cell surface after addition of virus. Approximately 30 min after addition of virus, discrete areas on the cell surface showed fluorescent staining, which coalesced to apical regions of the cell after approximately 40 min.

The initial event in the replicative cycle of an animal virus is the adsorption of the virus particle to the host cell plasma membrane (8, 30). This attachment involves specific interactions between the virion and the plasma membrane surface proteins and lipids capable of functioning as virus receptors (13, 15, 30). Virus binding can induce changes in the lateral distribution of cell membrane receptors by causing the formation of virus receptor patches in the plane of the plasma membrane (3, 14, 15). Attachment of influenza and Sendai viruses or lectins alters the fluidity of the membrane lipid bilayer of avian erythrocytes (31, 32). This effect requires the cross-linking of receptors and can be inhibited with microtubule-disrupting drugs (31, 32).

We investigated plasma membrane structural changes caused by the binding of the enveloped, negative-strand virus vesicular stomatitis virus (VSV) to tissue culture cells by using spin label electron spin resonance (ESR) methods. VSV binds to cells via an envelope-associated surface glycoprotein (G-protein) (4, 5, 6, 22). ESR spectra of a nitroxide derivative of stearic acid incor-

porated into the plasma membrane of intact BHK-21 cells indicate that the plasma membrane lipid bilayer becomes more rigid upon adsorption of VSV. This increase in rigidity can be inhibited by colchicine.

Ligands that are monovalent or have reduced valency are generally unable to induce clustering of receptors in the plane of the plasma membrane (12, 18, 23, 36). Cells infected with VSV produce a water-soluble glycoprotein that is derived from G-protein (20, 21, 28, 29). This soluble glycoprotein, G_s , appears to be monovalent (unpublished data). It is reported here that treatment of cells with G_s does not cause a change in plasma membrane lipid bilayer fluidity. When immunoglobulin G (IgG) directed against G-protein is added to cells pretreated with G_s , the plasma membrane lipid bilayer becomes more rigid. By measuring the distribution of fluorescently labeled VSV on the cell surface, it was found that bound virus formed discrete clusters on the cell surface and that these patches of bound virus moved to apical regions of the cell approximately 40 min after addition of virus. These results suggest that binding of VSV to cell plasma membrane receptors involved a colchicine-sensitive lateral rearrangement of receptors in the plane of the plasma membrane.

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MATERIALS AND METHODS

Cells. Monolayers of BHK-21 cells were grown on Falcon (Falcon Plastics, Oxnard, Calif.) plastic tissue culture flasks in reinforced Eagle medium containing 10% tryptose-phosphate broth and 10% calf serum (16).

Virus. Stocks of the Indiana serotype of VSV were maintained and assayed on BHK-21 cells (7). Virus was prepared by infecting BHK-21 cells with VSV suspended in Eagle medium at a multiplicity of infection equal to 0.1 PFU per cell. After a 2-h adsorption period, the inoculum was removed and replaced with Dulbecco medium. The supernatant from the infected cells was removed approximately 14 h after infection at 37°C. Cell debris was removed by low-speed centrifugation ($2,500 \times g$, 20 min). Virus was recovered from the clarified supernatant medium by high-speed centrifugation ($25,000 \times g$, 2 h). The pelleted virus was purified by density gradient centrifugation on linear 5 to 40% potassium tartrate gradients (26).

Preparation of soluble glycoprotein. The supernatant medium recovered after virus pelleting was brought to 70% saturation with ammonium sulfate. The precipitated protein was recovered by centrifugation, and the resulting pellet was suspended in water and dialyzed against 10 mM Tris-0.150 M NaCl (pH

7.4) buffer (TBS). The dialyzed protein mixture was subjected to high-speed centrifugation ($100,000 \times g$, 6 h) to remove insoluble aggregates. Final purification was achieved by Sepharose 4B gel filtration chromatography and eluting with TBS.

Spin labeling. The cells were harvested by gentle scraping. They were washed with phosphate-buffered saline, and $10 \mu\text{l}$ of 1-mg/ml C_5 stearic acid spin label [2-[carboxypropyl]-4,4 dimethyl-2-tridecyl-3-oxazolidinyloxy] (structure shown in Fig. 1) in ethanol was added to the cell pellet. The cells were immediately washed with phosphate-buffered saline and aspirated into a capillary pipette. ESR spectra were recorded with a Varian E-12 ESR spectrometer. All spectra were recorded at room temperature.

Fluorescent labeling and fluorescence microscopy. Fluorescein isothiocyanate at a final concentration of $100 \mu\text{M}$ was added to a 2-mg/ml suspension of VSV in 10 mM sodium phosphate buffer (pH 8.5). After a 12-h incubation period at 4°C, the virus suspension was dialyzed against phosphate-buffered saline to remove the unbound label, and the labeled virus was repurified as described above. The labeled virus in excess was added to cells grown on glass cover slips. At 5-min intervals after addition of virus, the cover slips were mounted on glass microscope slides and washed with phosphate-buffered saline, and the

FIG. 1. ESR spectra of the C_5 spin label incorporated into the plasma membrane lipid bilayer of BHK-21 cells. The structure of the spin label is shown in the upper left. The upper spectrum is that of the spin label incorporated into control BHK-21 cell plasma membranes of intact cells. The lower spectrum is that of the spin label in the plasma membrane of intact cells which had been treated with 100 PFU of VSV per cell at 37°C for 30 min before being harvested and spin labeled. The distance between the outermost peaks in the spectrum is defined as $2A'_{zz}$. The outermost peaks of the spectra were amplified to aid in the determination of $2A'_{zz}$.

cells were examined for bound fluorescence (9). Incubations with virus were done at 37°C, and microscopy was done at room temperature.

Reagents. Fluorescein isothiocyanate was obtained from Sigma Chemical Co., St. Louis, Mo. Spin label was purchased from Syva, Palo Alto, Calif. All other chemicals used were the highest grade obtainable. Anti-VSV G-protein IgG was isolated by ion-exchange chromatography on DEAE-cellulose (27) from rabbit antiserum against purified VSV G-protein. Rabbit antiserum was kindly provided by John Lenard, College of Medicine and Dentistry of New Jersey, The Rutgers Medical School.

RESULTS

Figure 1 shows the ESR spectra of the C_5 label incorporated into the plasma membrane of untreated BHK-21 cells and of BHK-21 cells which had been treated with 100 PFU of VSV per cell in Eagle medium without serum at 37°C for 30 min before harvesting and spin labeling. As discussed previously in detail, it appears that the ESR signal detected from C_5 -labeled intact BHK-21 cells is due to spin label incorporated into the plasma membrane lipid bilayer and not to spin label incorporated into cytoplasmic membranes (25). The binding of VSV caused a slight, but easily detectable, increase in the distance between the outermost peaks of the spectrum ($2A'_{zz}$ defined in Fig. 1), indicating an increase in the average rigidity of the membrane lipid bilayer (17, 19, 24). The ESR spectra therefore suggest that binding of VSV to BHK-21 cells results in a structural change in the plasma membrane lipid bilayer. This structural change reaches a maximum approximately 30 min after addition of virus.

BHK-21 cells were treated with multiplicities of VSV, ranging from 10 to 100 PFU per cell, and spin labeled after a 30-min adsorption period. The increase in plasma membrane bilayer rigidity caused by the addition of virus is multiplicity dependent, requiring 50 PFU per cell to produce a measurable increase in the rigidity of the plasma membrane, and saturates at approximately 100 PFU per cell.

The VSV virion contains many copies of G-protein. It is therefore possible that each VSV particle binds to several G-protein receptors on the cell surface. To measure the effects of ligand valency on membrane fluidity, these experiments were repeated with the monovalent derivative of G-protein, G_s . Addition of 1 mg of G_s per ml in phosphate-buffered saline did not cause a measurable change in bilayer rigidity. However, when anti-G-protein IgG, which specifically precipitates G_s (33), was added to cells pretreated with G_s , the ESR spectra of the C_5 probe incorporated into the membrane indicated that the plasma membrane bilayer became more rigid

(Table 1). These results and the fact that the ratio of viral lipid to plasma membrane lipid in these experiments is very small indicate that the observed changes in the ESR spectrum of spin-labeled plasma membranes are not due to diffusion of spin label into the envelopes of the virus particles.

Preincubation of the cells with 10 μ M colchicine in Eagle medium for 30 min at 37°C prevented both virus-induced changes in membrane bilayer fluidity and alteration of membrane rigidity due to the cross-linking of membrane-bound G_s by IgG (1). The increase in plasma membrane bilayer rigidity could be reversed by the addition of colchicine to VSV-treated cells. Preincubation of the cells with the metabolic inhibitors dinitrophenol and F^- (1 mM dinitrophenol-10 mM NaF in phosphate-buffered saline) did not significantly inhibit the increase in bilayer rigidity caused by VSV.

The results of the ESR experiments suggest that binding of VSV and the cross-linking of membrane-bound G_s by anti-G-protein IgG cause a change in the fluidity of the BHK-21 plasma membrane lipid bilayer. The multisite binding of a polyvalent ligand may cross-link plasma membrane receptors, causing a change

TABLE 1. Effects of VSV, G_s , anti-VSV G-protein IgG, and colchicine on the fluidity of the BHK-21 cell plasma membrane^a

Expt	$2A'_{zz}$ (experiment) - $2A'_{zz}$ (control)
	(Gauss)
BHK	0.0
BHK + VSV	1.3
BHK	0.0
BHK + G_s	0.0
BHK	0.0
BHK + IgG	0.3
BHK + G_s + IgG	1.0
BHK	0.0
BHK + colchicine	0.0
BHK + colchicine + VSV	0.3
BHK + VSV + colchicine	0.3
BHK	0.0
BHK + G_s	0.0
BHK + G_s + IgG	1.0
BHK + G_s + colchicine + IgG	0.3

^a The order of the reagents in each entry (e.g., BHK + G_s + IgG) represents the order in which the various reagents were added to the cells before harvesting and spin labeling. A change in $2A'_{zz}$ (experiment) - $2A'_{zz}$ (control) greater than 0.3 Gauss indicates that the treatment in the table entry caused an increase in the rigidity of the cell plasma membrane lipid bilayer.

in the lateral distribution of proteins in the plane of the plasma membrane.

VSV labeled with fluorescein isothiocyanate was added to cells at 37°C, and the pattern of fluorescent staining on the periphery of the unfixed cells was examined at 5-min intervals after addition of virus. Few areas of localized fluorescence were observed before 20 min after addition of fluorescein isothiocyanate-labeled VSV. However, after 20 min, the fluorescence was in the form of patches around the periphery of the cell. The capping appeared to be complete by 40 min. (Relatively little intracellular fluorescence was observed 40 min after infection.) This result and observations by others (3, 14) suggest that virus binding can induce the lateral rearrangement of cell surface proteins.

DISCUSSION

The data presented indicate that binding of VSV to BHK-21 cells causes a measurable increase in the rigidity of the plasma membrane lipid bilayer as detected with a nitroxide derivative of stearic acid. In addition, the data suggest that this increase in rigidity is sensitive to the microtubule-disrupting drug colchicine.

Lyles and Landsberger (31, 32) have shown that the fluidity of avian and amphibian erythrocyte membrane lipid bilayers increases upon agglutination by influenza and Sendai virions or lectins and that this change in bilayer fluidity requires multisite binding and can be inhibited by colchicine (31, 32). The change in bilayer fluidity observed upon binding of VSV to BHK-21 cells is in the opposite direction from that observed for nucleated erythrocyte systems. This probably reflects the differences in lipid and protein composition and in lipid-lipid and lipid-protein interactions in the plasma membranes and not differences in the mechanisms underlying the observed changes in the plasma membranes upon adsorption of virus. Unfortunately, at present little is known about these interactions; thus, it is not possible to give a description of the mechanisms underlying these results. The observed changes in membrane bilayer fluidity in both systems do require binding of multivalent ligands and are sensitive to colchicine, suggesting that a similar mechanism may be involved. Both the binding of influenza and Sendai viruses to avian erythrocytes and the binding of VSV to BHK-21 cells appear to involve the lateral rearrangement of membrane receptors, as reflected by the requirement that (i) the receptors be cross-linked and (ii) the apparent dependence of the binding-induced bilayer fluidity change upon microtubule-like systems which may be involved in receptor mobility in many membrane systems (10, 31, 32, 35).

The observation that VSV bound to the surface of the BHK-21 cell membrane can form patches and caps provides additional evidence for the hypothesis that virus binding involves lateral redistribution of cell surface receptors. It has been postulated that membrane structural changes such as receptor rearrangement are involved in endocytosis (2, 36), the probable mechanism for VSV penetration (8, 11, 34). At present, a distinction cannot be made between a direct effect of receptor rearrangement on bilayer fluidity and some event subsequent to adsorption, such as endocytosis. However, since relatively little intracellular fluorescence was observed during the time periods when capping and patching occurred, and since the virus-induced increase in plasma membrane bilayer rigidity occurred in the presence of a metabolic inhibitor (NaF) which inhibits endocytosis (37), it appears that the alteration of membrane fluidity observed upon addition of virus is due to the cross-linking of cell membrane receptors, rather than to a metabolically coupled event such as endocytosis.

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