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LIPID AND PROTEIN CONTRIBUTIONS TO THE MEMBRANE SURFACE POTENTIAL OF VESICULAR STOMATITIS VIRUS PROBED BY A FLUORESCENT pH INDICATOR, 4-HEPTADECYL-7-HYDROXYCOUMARIN

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The surface potential of membranes of vesicular stomatitis virus and liposomes was determined by shift of ionization over a wide pH range of the membrane-inserted fluorophore, 4-heptadecyl-7-hydroxycoumarin. Incorporation into sonicated vesicles of negatively charged phosphatidylserine markedly increased the surface potential of uncharged phosphatidylcholine, but no significant effect on surface potential was produced by polar but uncharged glucocerebroside incorporated in phosphatidylcholine vesicles. The membrane of vesicular stomatitis virus was found to have a moderately high surface potential. Contributing to this viral membrane surface potential were glycoprotein spikes and phospholipid headgroups as determined by lowered charge after treatment of intact virions with thermolysin to remove glycoprotein or phospholipase C to remove phospholipid headgroups. The role of viral glycoprotein was confirmed by demonstrating increased surface charge of vesicles reconstituted with both viral glycoprotein and lipids compared with vesicles reconstituted with viral lipids alone. An unexpected finding was the large contribution to surface potential of cholesterol present in viral membrane. Increasing cholesterol concentration in virions by interaction with cholesterol-complexed serum lipoproteins resulted in a marked decrease in surface potential, whereas 75% depletion of virion cholesterol by interaction with sphingomyelin-complexed serum lipoproteins resulted in a significant increase in virion membrane surface potential. Although removal of glycoprotein spikes or depletion of cholesterol causes reduction in infectivity of vesicular stomatitis virus, no direct correlation could be found between alteration in surface charge and infectivity.

Introduction

Most biological membranes are electrostatically charged owing to ionogenic groups of membrane proteins, phospholipids, and glycolipids at the cell surface. In most cells, these ionogenic groups produce a net negative charge at physiological pH [1,2]. The surface potential of cell membranes results both from these fixed charge groups and also from the unequal adsorption of anions and cations interacting with the cell surface [1]. although the exact significance of charge at membrane surfaces is not clearly understood, it is apparent that charge and potential at the cell surface influence cell recognition functions such as cell-cell adhesion [1], interaction of cells with various proteins [3] (including enzymes and viruses), and possibly the fusion of two interacting membranes [4]. This correlation between surface charge and function has led to many attempts to measure membrane elec-

Abbreviations: VSV, vesicular stomatitis virus; BHK-21 cells, baby hamster kidney cells, clone 21; pfu, plaque-forming units; PC, phosphatidylcholine; PS, phosphatidylserine.

trical potential quantitatively. These methods include measuring membrane conductance caused by small amounts of ionic and zwitterionic surfactants [5] and distribution of paramagnetic amphiphiles between membrane and the bulk phase measured by electron spin resonance spectroscopy [6]. Estimates of surface potential have also been made by measuring the electrophoretic mobility of liposomes, cells and organelles [1,7,8].

A number of pH-sensitive chromophores has also been used for studying the interfacial potential of various energy-transducing membranes, artificial lipid bilayers, and micellar complexes [9,10]. Fluorescent phenolic chromophores, such as umbelliferone (7-hydroxycoumarin), when attached to a long hydrocarbon chain, can intercalate into lipid bilayers [11–14]. These probes show pH-dependent spectral changes and thus have been utilized to measure change density at lipid-water interfaces. In all cases, the measured surface potential agreed well with surface potentials predicted by the Gouy-Chapman equation [5,6,13].

In this communication, we describe the use of a fluorescent pH indicator, 4-heptadecyl-7-hydroxycoumarin, to measure the surface potential of the membrane of vesicular stomatitis virus (VSV). VSV is a negative-strand RNA virus with a lipid bilayer envelope derived by budding from the host cell plasma membrane [15]. The membrane of this virus has been well characterized and serves as an excellent model system for studying a biological membrane much less complex than cell membranes [16]. The VSV membrane is composed of common phospholipids, cholesterol, and a small amount of glycolipid; there are only two proteins, an integral glycoprotein which protrudes from the external surface and a peripheral matrix protein which lines the inner surface of the bilayer in association with the underlying nucleocapsid [17–19]. The glycoprotein spike is the organ for attachment of virus to host cell surfaces; it has also been shown that viral membrane cholesterol contributes to infectivity of the virus, but phospholipid headgroups apparently do not [20–22].

The present studies were designed to provide information on the surface potential of the relatively simple VSV membrane and the degree to which the various membrane components contribute to the surface potential. We also hope to gain some insight into the role of membrane surface potential in physical and biological properties concerned with membrane-membrane interaction leading to viral infection of the host cell. Measurements of surface potential were made after perturbation of the viral membrane with a protease to remove glycoprotein spikes, with phospholipase C to remove phospholipid headgroups, by enrichment or depletion of membrane cholesterol, and by reconstitution of extracted viral lipids and glycoprotein into lipoprotein vesicles.

Materials and Methods

The Indiana serotype (San Juan strain) of VSV was prepared by infecting confluent monolayers of BHK-21 cells with cloned virus at a multiplicity of 0.1 pfu/cell. Homogeneous bullet-shaped particles were harvested at 18 h post-infection and were purified by sequential cycles of differential, rate zonal, and equilibrium centrifugation as described previously [23]. To remove the glycoprotein spikes, VSV (1 mg/ml) in 10 mM Tris-HCl buffer (pH 7.4) was exposed to 15 units of thermolysin (EC 3.4.24.4, Sigma Corp., St. Louis, MO) at 39°C for 1 h and then pelleted at $100\,000 \times g$ for 80 min through a pad of 50% glycerol; the pellet of spikeless virions was suspended in 50 mM KCl flor fluorescence measurements. To hydrolyze the phospholipid headgroups in the external layer of the VSV membrane, phospholipase C (*Clostridium welchii* type I, Sigma, EC 3.1.4.3) was added to VSV (0.5 mg/ml) at a concentration of 0.75 units of enzyme per mg of viral protein; after incubation at 37°C for 3 h, the phospholipase C-treated VSV was pelleted through a 50% glycerol pad by centrifugation at $100\,000 \times g$ for 80 min [24]. Cholesterol was depleted from the VSV membrane by incubating virions with lipoproteins present in 10% fetal calf serum in basal medium (Eagle) enriched with bovine brain sphingomyelin, as described elsewhere [22]; incubation of VSV for 24 h with this sphingomyelin-enriched serum-medium mixture resulted in at least 75% depletion of cholesterol from the virion membrane. To enrich the membrane cholesterol content, purified VSV was incubated with lipoproteins in the same serum-medium mixture but in this case saturated with cholesterol in addition to phospholipids [25];

by this technique, the cholesterol concentration of the VSV membrane could be enriched by 50 mol% to a lipid concentration of approx. 60% mol cholesterol.

The fluorophore 4-heptadecyl-7-hydroxycoumarin was obtained from Molecular Probes, Junction City, Oregon and was dissolved in tetrahydrofuran to a final concentration of 1 mM. Aliquots of this solution were injected into suspension of VSV at a molar ratio of 80 viral lipid molecules to 1 fluorophore molecule. The fluorescence spectra of 4-heptadecyl-7-hydroxycoumarin were recorded in a Perkin-Elmer MPF-3 fluorescence spectrophotometer. Fluorescence intensity of the virus-fluorophore mixture increased with time. After incubation for 2 h at 37°C (well after reaching the plateau of fluorescence intensity), the virions were pelleted or purified by gradient centrifugation to remove the unbound fluorophore. The pelleted virus was then resuspended in 50 mM KCl and 0.01 M NaOH was added to adjust the solution to the desired pH, measured by a pH electrode. Both ionized and unionized forms of 4-heptadecyl-7-hydroxycoumarin are present in such suspensions [13], but the two forms can be distinguished by their different excitation and emission spectra; the excitation maxima are 325 nm and 375 nm for the unionized and ionized forms, respectively, and emission maxima are 415 nm and 450 nm for un-ionized and ionized forms, respectively. Titration of the fluorescent probe incorporated into the VSV membrane was performed in the pH range of 6.0 to 10.5. To determine the degree of dissociation of the fluorophore at different pH values, and in order to compare results between various experiments, we measured the area integrated under the 325 nm and 375 nm peaks and used the ratio calculated between the two peaks. The apparent pK values (pK_a) were obtained from the curve describing the degree of dissociation (α) as a function of the pH. Apparent pK (pK_a) is equivalent to pH, where degree of dissociation (α) is 0.5 [11,13].

It is quite likely that a certain fraction of the fluorophore incorporated in the viral or model membrane may partition into the inner leaflet of the bilayer. However, preliminary studies had shown that only a small fraction of the fluorophore resides in the inner leaflet for significant exposure to the bulk pH of the medium, presumably owing to rapid trans-bilayer movement of the probe.

The surface potential (Ψ) was calculated from the shift in pK introduced by changes in surface charge (pK_{ch}) relative to pK_o for the uncharged surface, giving rise to the following equation:

$$pK_{ch} = pK_o - (\Psi F/2.3\,RT) \quad (1)$$

where R, F, and T are the gas constant, Faraday constant and the absolute temperature, respectively. This equation can be rewritten in the form:

$$\Psi = -(pK_{ch} - pK_o)2.3\,RT/F \quad (2)$$

The value for pK_o was obtained by using small unilamellar vesicles of egg phosphatidylcholine, which is uncharged in this pH range [26].

Egg phosphatidylcholine was purchased from Makor Chemicals, Jerusalem, Israel; dicetyl phosphate and phosphatidylserine were obtained from Sigma Chemical Co., St. Louis, MO. Glucocerebroside was a gift from T.E. Thompson, University of Virginia. Small, unilamellar vesicles were prepared by sonication under nitrogen of the desired lipids [20]. The sonicated vesicles were centrifuged at $100\,000 \times g$ for 60 min to remove the multilamellar vesicles. Vesicles reconstituted from the lipids and glycoprotein of VSV extracted with octylglucoside were prepared by detergent dialysis according to the method of Petri and Wagner [27]. Measurements of surface potential of sonicated vesicles or vesicles reconstituted from VSV lipids and glycoprotein were performed in a manner similar to that of intact VSV membranes, but for the vesicles the lipid to fluorescent probe ratio was 200 : 1 rather than 80 : 1.

Results and Discussion

Fig. 1 compares the excitation spectra of 4-heptadecyl-7-hydroxycoumarin incorporated into sonicated egg phosphatidylcholine vesicles (Fig. 1A) or in intact VSV membrane (Fig. 1B) over a pH range of 5.8 to 10.5. The excitation spectra were recorded by fixing the emission wavelength at 450 nm. The ratio of the area under the two peaks was used to calculate pK_a values as de-

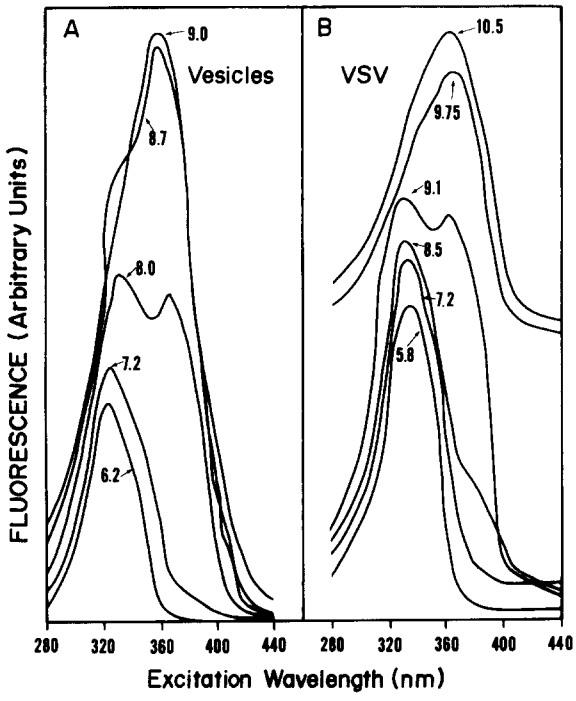

Fig. 1. Excitation spectra of the ionized and un-ionized forms at different pH values (→) of 4-heptadecyl-7-hydroxycoumarin partitioned into the membranes of (A) egg phosphatidylcholine (100 nmol/ml) or (B) VS virions (200 μg/ml). The emission wavelength was fixed at 450 nm and the spectra were scanned between 280 and 420 nm. The pH of each suspension was altered by periodic addition of 0.01 M NaOH and the pH measured by an electrode. Suspensions of small sonicated egg PC vesicles in 50 mM KCl and of VSV were prepared as described in Materials and Methods. The ratio of lipid to fluorophore molecules was 200:1 for PC vesicles and 80:1 for the VS virus suspension. Unbound probe was removed from VSV suspensions as described in Materials and Methods. As designated for each spectrum, PC vesicles were scanned at pH levels of 6.2 to 9.0 and VS virions were scanned at pH 5.8 to 10.5. The excitation peaks for the fluorophore inserted in egg PC vesicles was 325 nm for the un-ionized form and 375 nm for the ionized form. The specta for VSV (B) at pH 9.75 and pH 10.5 were recorded at one-third the sensitivity of that for other determinations.

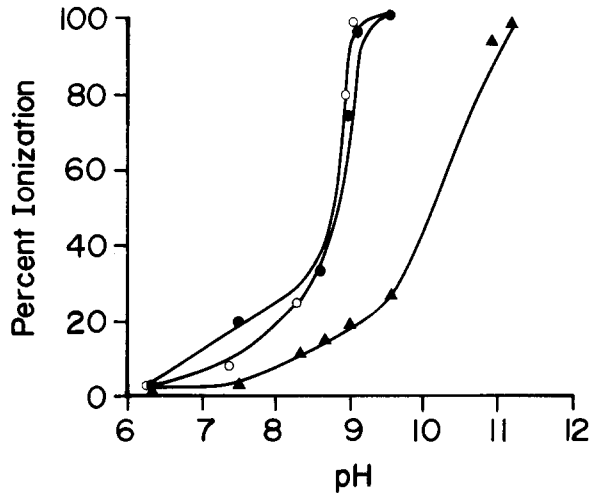

Fig. 2. Comparative mode of ionization of 4-heptadecyl-7-hydroxycoumarin present in sonicated lipid vesicles composed of egg PC (○), egg PC: glucocerebroside in 80:20 molar ratio (●) or egg PC: brain PS in 50:50 molar ratio (▲). The small unilamellar lipid vesicles were prepared by sonication as described elsewhere. The degree of ionization was measured by integrating the areas under the 325 nm peak and the 375 nm peaks of the excitation spectra. The total peak area of the whole spectrum was measured and the major peak was separated from the total spectrum. The peak area of the major species was calculated and was subtracted from the total peak area to obtain the peak area of the minor species. The degree of ionization was then determined from the peak area for the ionized compared to the un-ionized species.

scribed for the baseline pK_o value for egg phosphatidylcholine vesicles, of which the degree of dissociation (α) was 1.0 at pH 9.77 and zero at pH 6.4. The values for pK_a were obtained by plotting α as a function of pH and these values are shown in Fig. 2 for egg phosphatidylcholine, egg phosphatidylcholine: : glucocerebroside (80 : 20), and egg phosphatidylcholine: brain phosphatidylserine (50 : 50). The pK_o value for egg PC vesicles was 8.65 whereas the presence of PS in PC/PS vesicles at a molar ratio of 1 : 1 shifted the pK_{ch} to 10.15. When glucocerebroside was present in egg PC vesicles at a molar ratio of 1 : 4, the pK_{ch} value was found to be 8.7 which is quite close to the baseline pK_o value for egg PC alone. Surface potentials (Ψ) for PC vesicles and PC/PS vesicles, calculated from pK values according to Eqn. 2, are shown in Table I. As noted, introduction of a charged phospholipid (PS) into the vesicle bilayer has a large effect on the surface potential as monitored by the fluorophore 4-heptadecyl-7-hydroxycoumarin. The potential was increased from 0 mV for PC vesicles to 88 mV for PC/PS vesicles. In contrast, introducing glucocerebroside, an uncharged (but very polar) molecule, into PC vesicles has a minimal effect on surface potential.

The fluorophore, 4-heptadecyl-7-hydroxycou-

TABLE I

SURFACE pK AND ELECTRICAL SURFACE POTENTIAL (Ψ) COMPARED FOR MEMBRANES OF INTACT AND ALTERED VS VIRIONS AND RECONSTITUTED VESICLES AS WELL AS COMPARATIVE INFECTIVITY OF VS VIRIONS WITH INTACT OR ALTERED MEMBRANES

The fluorescence spectra over a wide pH range were determined for 4-heptadecyl-7-hydroxycoumarin partitioned into each membrane; the pK and Ψ values were calculated from Eqns. 1 and 2 described in Materials and Methods.

Membrane	pK	Ψ (mV)	Fractional infectivity [i]
PC vesicles [a]	8.65	0	
PC/PS vesicles [b]	10.15	−87.7	
Intact VSV	9.5	−49.6	1.0
VSV + thermolysine [c]	8.85	−11.7	0.00001
VSV + phospholipase C [d]	8.65	0	0.6
VSV cholesterol-enriched [e]	8.8	−8.7	1.0
VSV cholesterol-depleted [f]	10.15	−87.7	0.02
VSV lipid vesicles [g]	9.05	−23.4	
VSV lipid/G vesicles [h]	9.45	−46.7	

[a] Sonicated egg phosphatidylcholine vesicles.

[b] Sonicated egg phosphatidylcholine + egg phosphatidylserine (1 : 1 molar ratio).

[c] Glycoprotein spikes removed from VSV by exposure to 15 units of thermolysin.

[d] Phospholipid headgroups removed from VSV by exposure to 0.75 units of phospholipase C.

[e] VSV membrane cholesterol enriched by approx. 50 mol% by interaction with serum lipoproteins saturated with cholesterol.

[f] VSV membrane cholesterol depleted by approx. 75 mol% by interaction with serum lipoproteins complexed with sphingomyelin.

[g] Vesicles created by detergent dialysis of total lipids extracted from VSV.

[h] Lipoprotein vesicles reconstituted by detergent dialysis of glycoprotein and lipids extracted from VS virions and mixed at a ratio of 1 : 100.

[i] Infectivity values are recorded from results of plaque-assay determinations previously recorded for VS virions treated with thermolysin [28], phospholipase C [20], and serum lipoproteins [22,25]. Levels of viral infectivity were determined by plaque assay on L-cell monolayers. The data are recorded as the fractional reduction of infectivity of the control (intact virions) which had a baseline titer of $2 \cdot 10^9$ pfu/ml.

marin, was found to be incorporated quite readily into the membrane of VSV and, as shown in Fig. 1B, the excitation spectra varied greatly at different pH values. The probe was un-ionized at pH 5.8 and was completely ionized at pH 10.5.

We next set out to determine what effect the various lipids and glycoprotein of the VSV membrane had on the ionization patterns of the fluorescent probe, 4-heptadecyl-7-hydroxycoumarin. For this purpose, we compared intact virions with those in which glycoprotein spikes were removed by thermolysin, phospholipid headgroups were hydrolyzed by phospholipase C, or cholesterol was depleted or enriched in the virion membrane by interaction with serum lipoproteins. Fig. 3 shows that treatment of VS virions with thermolysin or phospholipase C markedly affected ionization of the fluorescent probe in the VSV membrane; the pK values were reduced in both cases compared to the pK of untreated control virus. These data indicate that the externalized glycoprotein and phospholipid headgroups protruding from the membrane surface significantly influence the viral membrane ionization potential.

Cholesterol represents another important constituent of the VSV membrane [22] and Fig. 3 shows that cholesterol content also significantly affects membrane ionization potential. In these experiments, the amount of cholesterol in the VSV membrane was depleted by approx. 75% by interaction with serum lipoproteins complexed with bovine brain sphingomyelin [22] or enriched by 50% above normal levels by reacting virions with cholesterol-saturated serum lipoproteins [25] (data not shown). The ionization of the fluorophore in these cholesterol-enriched and cholesterol depleted virions is depicted in Fig. 3. As noted, the pK value of the probe was considerably decreased in the membrane of cholesterol-enriched VSV and markedly increased in the membrane of cholesterol-depleted VSV compared to the membrane of untreated VSV controls, the cholesterol content of which was approx. 35 mol%.

The effect of glycoprotein on the ionization potential of the viral membrane was also determined by measuring the surface potential (Ψ) of the fluorophore inserted into vesicles created from lipid alone extracted from VS virions compared with lipoprotein vesicles reconstituted by the detergent-dialysis method [27] from lipids and glycoprotein extracted by octylglucoside from VSV virions. At least 90% of the glycoprotein in recon-

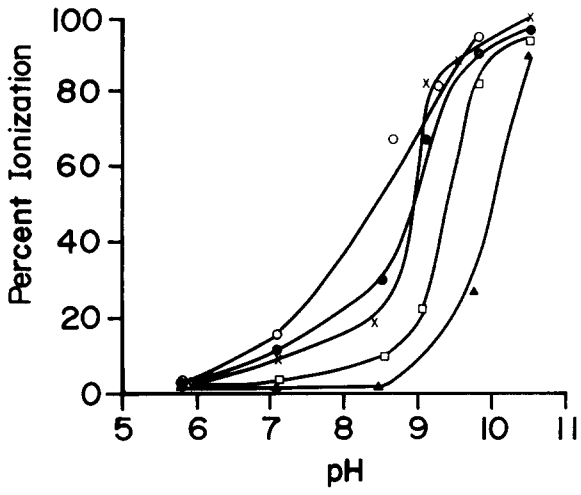

Fig. 3. Comparative mode of ionization of 4-heptadecyl-7-hydroxycoumarin present in intact VS virions (□), thermolysin-treated VS virions (●), phospholipase C-treated VS virions (○), cholesterol-enriched VS virions (×), and cholesterol-depleted VS virions (▲). The removal of glycoprotein spikes by thermolysin and of phospholipid headgroups by phospholipase C are described in Materials and Methods, as are the enrichment by 50% and depletion by 75% of cholesterol from VS virions after interaction with serum lipoprotein complexed with cholesterol or bovine brain sphingomyelin, respectively. The inital ratio of viral lipid to fluorophore was 80 : 1 and the degree of ionization of the probe in each virus preparation was measured by integration of the two peaks as described in the text. Excess unbound probe was removed as described in Materials and Methods.

stituted lipoprotein vesicles protrudes from the external surface of the vesicle bilayer [27]. Data shown in Table I demonstrates that vesicles made of VSV lipids alone have a slightly negative surface charge potential of −23 mV compared with a surface charge potential of −47 mV for VSV lipid vesicles in which the viral glycoprotein has inserted itself. This surface potential of the reconstituted lipid-glycoprotein bilayer is almost identical to that of the intact VS virion membrane (see Table I). These results support the evidence for the contribution to surface charge of the VSV glycoprotein obtained from experiments showing the effect of thermolysin on the surface charge of the VS virion membrane.

Table I summarizes all the date obtained in these experiments by comparing the surface potential (Ψ, derived from Eqn. 2) of sonicated phospholipid vesicles, intact VS virions, and liposomes reconstituted from VSV lipids and glycoprotein with that of VS virion membranes in which glycoprotein and phospholipid headgroups have been removed and cholesterol content altered by depletion or enrichment. Treatment of virions with either phospholipase C or with thermolysin reduced the negative charge of the virion membrane resulting in a significant decrease in surface potential. Virions treated with phospholipase C exhibited no negative surface potential whatsoever compared to the intact charge of −49.6 mV. Removal of glycoprotein spikes by treatment with thermolysin resulted in quite a significant effect but less than that of phospholipid headgroups removed by phospholipase C. Of considerable interest was the finding that enrichment of virion cholesterol by approx. 50 mol% also reduced the negative surface potential of virions to −9 mV, a level equivalent to that of spikeless virions. Depletion of virion cholesterol by approx. 75%, on the other hand, markedly increased the negative surface potential of the membrane from −50 mV to −88 mV.

Also shown in Table I are data from earlier experiments [20,22,25,28] showing the comparative infectivity of VSV following treatment with phospholipase C [20] or thermolysin [28] and following depletion [22], or enrichment [25] of virion cholesterol. Removal of phospholipid headgroups by phospholipase C or cholesterol enrichment had no significant effect on infectivity determined by plaque assay of virus on monolayers of L cells [20,22]. Depletion of cholesterol from the virion membrane by approx. 75% reduced VSV infectivity by a limited but quite significant degree of almost 100-fold [22]. The marked loss in infectivity caused by thermolysin removal of glycoprotein spikes reflects the complete loss of the viral cell-attachment organ [28] rather than just alteration in surface charge.

The work presented in this communication demonstrated that membrane lipids and glycoprotein both contribute to the electrical surface potential of the VS virion membrane as measured by the ionization of 4-heptadecyl-7-hydroxycoumarin present in the viral bilayer. Gonzales-Ros et al. [29] recently reported that the apparent pK of the same fluorescent probe was influenced by the nature of the phospholipids and the presence of

acetylcholine receptor, an integral glycoprotein, in the membranes of *Torpedo californica*. It is not clear whether the VSV glycoprotein, which is rich in sialic acid residues, contributes negative charges directly or by causing rearrangement of membrane lipids. However, similar reduction in negative charge was induced by proteolysis of glycoprotein using thermolysin or by hydrolysis of phospholipid headgroups using phospholipase C; in both cases, the resulting surface potential mapped to a similar value. Any correlation of reduction in negative surface charge to viral infectivity is questionable because proteolysis of glycoprotein reduces infectively drastically whereas phospholipid headgroup removal has no significant effect on infectivity.

The amount of cholesterol in the virion membrane appears to have a large effect on surface potential. Reducing the level of cholesterol below 75% of that normally present in the VS virion membrane increased the negative surface potential, whereas cholesterol enrichment reduced the negative surface potential markedly. This effect of cholesterol content on surface potential of the virion membrane does not appear to be due to interaction of cholesterol with the aromatic residue of the fluorophore. We could obtain no evidence that cholesterol alone affects the fluorescence properties of the coumarin probe. A neutral lipid like cholesterol by itself would not be expected to introduce fixed charges in the membrane. Therefore, one has to assume that cholesterol affects the organization of either glycoprotein or phospholipids or both in the lipid bilayer. We have preliminary evidence using the probe *trans*-parinaric acid that reduction in the level of cholesterol in the VSV membrane induces a broad phase separation in the virion bilayer (data not yet reported). However, no direct information is yet available on the expected effect of cholesterol on the organization of glycoprotein spikes in the virion membrane. The presumed interrelation of cholesterol content and membrane organization of glycoprotein as it relates to viral infectivity is yet to be determined. However, it seems clear that negative surface potential per se is not a significant determinant of VSV infectivity since marked reduction in negative charge by phospholipase C removal of phospholipid headgroups does not significantly reduce infectivity of the virus. Also of note is the finding that the surface potential of intact VSV membrane is very similar to that of vesicles reconstituted from VSV lipids and glycoprotein, indicative of the role of the glycoprotein in determining surface charge potential.

In conclusion, the data presented here appear to demonstrate that lipid composition and organization of the membrane of VS virions significantly affects the surface potential of the membrane. A similar effect is apparently exerted by the membrane glycoprotein of VSV, as judged by thermolysin removal and by the behavior of reconstituted glycoprotein-lipid vesicles. However, the correlation of these effects on surface potential to the assembly and biological activity of the VS virion membrane requires additional study.

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