Pyrene Phospholipid as a Biological Fluorescent Probe for Studying Fusion of Virus Membrane with Liposomes†

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Received July 10, 1987; Revised Manuscript Received September 17, 1987

ABSTRACT: We are using fluorescent endogenous phospholipids in virus membranes to study the factors that promote fusion on interaction with receptor membranes. To this end, vesicular stomatitis virus (VSV) grown in baby hamster kidney (BHK-21) cells was biologically labeled with fluorescent lipids, primarily phosphatidylcholine and phosphatidylethanolamine, derived from pyrene fatty acids. The pyrene lipids present in the virions showed a fluorescence spectrum typical of pyrene with an intense monomer and a broad excimer. Interaction of pyrene lipid labeled VSV with serum lipoproteins led to a spontaneous fast transfer of the small amount of pyrene fatty acids present in the envelope ($t_{1/2} \leq 7$ min), followed by a considerably slower transfer of pyrene phospholipids from the membrane of the virions ($t_{1/2} \geq 12$ h). Incubation of pyrene phospholipid labeled VSV with phosphatidylserine small unilamellar vesicles resulted in fusion at low pH (pH 5.0) as measured by the change in the excimer/monomer fluorescence intensity ratio. Fusion kinetics was rapid, reaching a plateau after 4 min at pH 5.0 and 37 °C. Only negligible fusion was noted at neutral pH or at 4 °C. Fully infectious virions labeled biologically with fluorescent lipids provide a useful tool for studying mechanisms of cell–virus interactions and neutralization of viral infectivity by specific monoclonal antibodies reactive with viral membrane glycoprotein.

The mode of penetration of host cells by viruses such as vesicular stomatitis virus (VSV)[1] has been only partially determined. Early electron microscopic studies (Heine & Schnaitman, 1971) showed that, under certain poorly defined conditions, the membrane of VSV can fuse with the surface cytoplasmic membrane of a host cell, presumably discharging the nucleocapsid of the virus into the cytoplasm. However, more recent studies have clearly demonstrated that VS virions are usually internalized by endocytosis in a manner akin to that of receptor-bound ligands (Matlin et al., 1982; Schlegel et al., 1982; Pastan & Willingham, 1983; White et al., 1983). During endocytosis, the intact virions appear to be engulfed in cytoplasmic vesicles; these endosomes are apparently rapidly acidified by their endogenous protein pump (Mellman et al., 1986). This pH reduction ostensibly initiates fusion of VSV membrane with endosome membrane, thereby releasing the transcribable viral nucleocapsid genome into the cytoplasm (White et al., 1983). This model of virus entry into the cell has been examined in vitro by studying the fusion at low pH of VS virions with liposomes and cell membranes probed by a spin-labeled phospholipid embedded in the virion envelope (Yamada & Ohnishi, 1986). In another study, Eidelman et al. (1984) demonstrated that VSV glycoprotein (G) partitioned into liposomes ("virosomes") can react at low pH with small unilamellar vesicles containing negatively charged phospholipids; this vesicle–vesicle interaction resulted in the appearance of large cochleate structures, and at low pH this fusion process can be studied by fluorescence energy transfer techniques (Eidelman et al., 1984). Although this study provides a good model for studying the fusion-promoting properties of the VSV G protein, other factors such as the VSV membrane matrix protein as well as lipid composition and bilayer orientation may well play a role; moreover, these vesicle–vesicle fusion experiments of Eidelman et al. (1984) were done at pH 3.3, which may not be physiological. Therefore, it seemed appropriate to devise a system where the virion membrane per se could be studied under more biological conditions.

The lipid composition of VS virions is well-defined, resembling but not identical with that of the host cell; phospholipids and cholesterol are the two major virion lipid components (Patzer et al., 1978, 1979). It is also possible to manipulate the lipid composition of the VSV envelope by growing the virus in mutant cell lines in medium supplemented with different fatty acids and sterol precursors (Pal et al., 1980). It has also been possible to incorporate in vivo various fluorescent lipids into the membrane of VS virions by supplementing the cell growth medium with fluorescent lipid precursors (Petri et al., 1981; Stoffel et al., 1978; Capone et al., 1983). In this paper, we report studies on the mode of incorporation of fluorescent lipids into the envelope of VSV by infecting BHK cells grown in the presence of pyrene fatty acids. Virions containing these biologically labeled fluorescent lipids provide an incisive means for studying the interaction of VS virions with model membranes as monitored by pyrene excimer formation.

Materials and Methods

Chemicals. 9-(1-Pyrenyl)nonanoic acid (PyC9), 10-(1-pyrenyl)decanoic acid (PyC10), 12-(1-pyrenyl)dodecanoic acid (PyC12), and 16-(1-pyrenyl)hexadecanoic acid (PyC16) were obtained from Molecular Probes (Junction City, OR) while

† This research was supported by Grant MV-9J from the American Cancer Society and by Grants AI-11112, AI-21652, and HL-17576 from the National Institutes of Health.

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[1] Abbreviations: VSV, vesicular stomatitis virus; BHK cells, baby hamster kidney cells; PyC9, 9-(1-pyrenyl)nonanoic acid; PyC10, 10-(1-pyrenyl)decanoic acid; PyC12, 12-(1-pyrenyl)dodecanoic acid; PyC16, 16-(1-pyrenyl)hexadecanoic acid; PyC10-keto, 10-(1-pyrenyl)-10-oxodecanoic acid; POPS,1-palmitoyl-2-oleoylphosphatidylserine; pfu, plaque-forming units; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; mAb, monoclonal antibody; moi, multiplicity of infection; IgG, immunoglobulin G; SPM, sphingomyelin; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane.

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10-(1-pyrenyl)-10-oxodecanoic acid (PyC10-keto) was obtained from Sigma Chemical Co. (St. Louis, MO). 1-Palmitoyl-2-oleoylphosphatidylserine (POPS) was obtained from Avanti Biochemicals (Birmingham, AL).

Cells and Virus. As described previously (Barenholz et al., 1976), BHK-21 cells were trypsinized gently and passed directly into Dulbecco's modified Eagle medium (Grand Island, NY) containing 5% calf serum, 10% tryptose phosphate broth, and 1% antibiotics. Various proportions (5–40 μg/mL) of fluorescent fatty acids in dimethyl sulfoxide (DMSO) were added to the growth medium so that the DMSO concentration was less than 1%. When cell growth was complete, generally after 3 days, the medium was removed, and the cell layer was washed once with phosphate-buffered saline (pH 7.4) and infected with VSV (Indiana serotype, San Juan strain) at a multiplicity of 0.1 pfu/cell. No fluorescent fatty acid was added to the medium after infection. The virus was harvested from the supernatant medium 18–21 h postinfection and purified by differential, rate zonal, and equilibrium centrifugation (Barenholz et al., 1976). The purified virions were pelleted by centrifugation, resuspended in PBS, and stored at −80 °C. The infectivity of the virus was measured by assaying for plaques formed on monolayers of L-929 cells (McSharry & Wagner, 1971).

Lipid and Protein Analysis. The total lipids of the virions were extracted by the method of Folch et al. (1957), and the phospholipids and neutral lipids were separated by thin-layer chromatography (TLC) on 250-μm silica gel G plates (Analytical Techniques, Newark, DE) using a solvent system of $CHCl_3$–MeOH–NH_4OH (65:25:5 v/v/v). The lipids in TLC plates were detected by UV light. The protein content in virus suspensions was estimated as described by Lowry et al. (1951). Liposomes used as model receptors for interaction with virion membrane were prepared by sonication of a phosphatidylserine suspension as described elsewhere (Wiener et al., 1983).

Phospholipase C Treatment. Phospholipase C (*Clostridium welchii* type 1, EC 3.1.4.3) from Sigma (St. Louis, MO) was dissolved in 20 mM Tris and 100 mM NaCl (pH 7.5) buffer. VS virions at a concentration of 0.5–1.0 mg/mL in the same buffer were made 1.3 mM with respect to $CaCl_2$ and were treated with 0.75 unit/mL phospholipase C at 37 °C for 3 h until all the phospholipids accessible to phospholipase C were hydrolyzed during that time (Patzer et al., 1978). The reaction was stopped by extraction of the lipids (Folch et al., 1957), and the lipids remaining in the chloroform phase after hydrolysis were analyzed by TLC as described above.

Fluorescence Measurements. Fluorescence emission intensity was measured in quartz cells with a Perkin-Elmer fluorescence spectrophotometer (MPF3). The pyrene-labeled lipids in virions were excited at 330 nm, and the uncorrected spectra were recorded at wavelengths from 360 to 530 nm. Estimation of pyrene lipids in the virus was done by reading the optical density of the lipid extract in ethanol at 345 nm and comparing with standard curves obtained with known amounts of fatty acids in ethanol. The kinetic processes involved in excimer formation of the pyrene chromophore have been reviewed extensively elsewhere (Galla & Hartmann, 1980; Lakowicz, 1981). The excimer to monomer fluorescence intensity ratio (E/M) was measured by exciting the pyrene-labeled virions at 330 nm; the fluorescence intensities of excimer and monomer emissions were determined at 470 and 385 nm, respectively.

RESULTS

Incorporation of Pyrene Lipids in the VSV Membrane. The pyrene chromophore provides a convenient means for continuously monitoring fusion between biological and model membranes (Galla & Hartmann, 1980; Lakowicz, 1981). Before studying the interaction of fluorescent lipid labeled VS virions with liposomes, it was necessary to investigate the mode of incorporation of the pyrene fatty acids into the virion envelope and the fluorescent properties of these lipids in the membrane. The chain length of the fatty acids could influence cell uptake and incorporation of resulting fluorescent phospholipids into the virion membrane. Therefore, monolayer cultures of BHK-21 cells were grown to confluency for 3 days in the presence of pyrene fatty acids varying in chain length. Cell growth was found to be normal in the presence of all these fluorescent fatty acids. The confluent monolayers were then washed thoroughly to remove unincorporated fatty acids and were then infected with VSV. No fluorescent fatty acids were added to the medium after VSV infection.

Table I: Incorporation of Pyrene Lipids in VSV Released from BHK Cells Grown in the Presence of Pyrene Fatty Acids of Different Chain Lengths[a]

type of fatty acid in growth medium	total pyrene lipids[b]	pyrene phospholipids[b]	pyrene neutral lipids[b]
PyC9	25.0	21.3	1.8
PyC10-keto	6.8	5.9	1.0
PyC10	27.5	21.6	1.6
PyC12	22.8	21.2	1.7
PyC16	26.1	21.2	1.8

[a] BHK cell monolayers grown to confluency in the presence of various pyrene fatty acids (30 μg/mL of growth medium) were infected with VSV at a multiplicity of 0.1 pfu/cell. The virions released in the medium were harvested at 21 h after infection and purified, and lipids were extracted and separated into phospholipids and neutral lipids by TLC. The amount of pyrene incoporated in each fraction was measured by optical density at 345 nm with suitable calibration curves for each fatty acid in which the molar extinction was determined. [b] Total pyrene lipids, phospholipids, and neutral lipids calculated as nanomoles per milligram of viral protein.

The degree of incorporation of pyrene fatty acids into the VSV membrane is shown in Table I. Pyrene fatty acids with chain lengths of C9, C10, C12, and C16 were all incorporated in the membrane of VSV to a significant extent, while the incorporation was markedly less for the PyC10-keto fatty acid. Total lipid extracted from viral particles was analyzed by TLC, and the amount of fluorophore present in both phospholipids and neutral lipids was measured. As shown in Table I, most of the pyrene fluorescence (>90%) was found in phospholipids while only 8% of the pyrene fluorescence was detected in neutral lipid fractions, mostly as free fatty acids.

The amount of pyrene lipids incorporated into the VSV membrane was also measured as a function of the amount of pyrene-labeled fatty acid added to the cell growth medium. In this experiment, BHK cells were grown to confluency in monolayer cultures in the presence of different amounts of PyC16 fatty acid. The cells were then infected with VSV in medium devoid of pyrene fatty acids, and virions released after incubation for 20 h at 37 °C were purified and assayed for their content of pyrene lipids and total protein. As shown in Figure 1, the pyrene content in the VSV envelope increased progressively when greater amounts of pyrene fatty acid were present in the preinfection BHK cell growth medium. The pyrene incorporated in the viral membrane was almost entirely associated with the phospholipid moiety; the residual fatty acids and neutral lipid fraction were not labeled significantly with pyrene (data not shown).

Figure 1 also shows that the yield of virus, as measured by protein concentrations, decreased significantly (~50%) with an increased in PyC16 fatty acid present in the preinfection BHK cell growth medium; this finding suggests that increasing

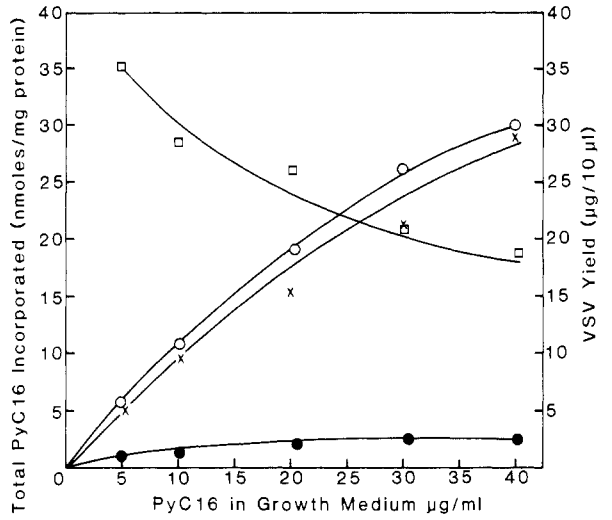

FIGURE 1: Uptake of fluorescent lipids by VSV as a function of the amount of pyrenylhexadecanoic acid (PyC16) added to the cell growth medium and the effect on virus yield. BHK cell monolayers were grown to confluency in the presence of various amounts of pyrenylhexadecanoic acid and then infected with VSV (moi $\simeq$ 0.1 pfu/mL). The virions were harvested at 20 h postinfection, and the lipids were extracted and separated by TLC into neutral lipids and phospholipids as described under Materials and Methods. Total pyrene incorporation in each fraction was measured by optical density at 345 nm. Total pyrene lipid uptake (○); neutral pyrene lipids (●); pyrene phospholipid (×); total VSV protein content harvested from BHK cells grown in different amounts of PyC16 (□).

the amount of fatty acid in the culture medium might have a deleterious effect on cells, which in turn would affect virus production. The infectivity of the PyC16-containing virions released from BHK cells grown in medium containing 40 μg/10 μL of PyC16 was also measured by plaque assays on L cell monolayer and compared with the infectivity of an identical amount of VSV released from BHK cells grown in the absence of pyrene fatty acids. It was observed that both control and pyrene lipid labeled virions had an identical infectivity titer of 3×10^{10} pfu/mL, which suggests that the presence of pyrene lipids in the virion membrane had no effect per se on the infectivity of the virus.

Nature and Bilayer Distribution of Pyrene Lipids in the VSV Membrane. PC, SPM, PE, and PS in ratios of ~24:24:31:18 are the major phospholipids present in the membrane of VSV grown in BHK cells (Patzer et al., 1978). Table II shows the overall composition and bilayer distribution of pyrene fluorescence in the various phospholipids in the membrane of VSV harvested from BHK cells grown in the presence of PyC16 fatty acid. PC and PE were the major lipids labeled with pyrene under such conditions, representing more than 85% of the total pyrene content in VSV. Only 5.3% of the total pyrene fluorophore was found in SPM and PS combined, although SPM and PS constitute 24% and 18%, respectively, of the total phospholipids in the VSV membrane (Patzer et al., 1978). As noted previously, neutral lipids accounted for only a small amount (8.7%) of the total fluorescence. In a few experiments, we noted a degraded product of pyrene lipid, which usually represented 5–8% of the total VSV pyrene fluorescence (data not shown).

The phospholipids in the envelope of intact VSV had previously been shown to be present in two pools, the head groups of one accessible to hydrolysis by phospholipase C and considered to reside in the outer monolayer and the other not accessible to phospholipase C, presumably present in the inner monolayer (Patzer et al., 1978). The choline-containing phospholipids are mainly located in the outer layer while the

Table II: Nature and Bilayer Distribution of VSV Membrane Lipids Labeled with 16-(1-Pyrenyl)hexadecanoic Acid (PyC16)[a]

lipid class	% total PyC16 lipids (unhydrolyzed control)	% pyrene lipid hydrolyzed by phospholipase C
PS and SPM	5.3	0
PC	42.2	95
PE	43.6	35
NL	8.7	

[a] Virions (1 mg/mL) grown in BHK-21 cells prelabeled with PyC16 fatty acid were treated with phospholipase C as described under Materials and Methods. The lipids were extracted from untreated and phospholipase C treated virions and separated into various phospholipid species and neutral lipids (NL) by TLC. The amount of pyrene incorporated in each species was measured by optical density at 345 nm. The amount of each phospholipid hydrolyzed was calculated by expressing the phospholipids of the enzyme-treated virus as a percent of an untreated control run in parallel. The percent hydrolyzed was then obtained by subtracting this value from 100%. Percent composition was calculated from the amount of each pyrene phospholipid in the untreated control.

inner layer of the VSV membrane is mainly composed of aminophospholipids (Patzer et al., 1978). The bilayer distribution of VSV phospholipids labeled with PyC16 fatty acid was examined by comparing by TLC the fluorescent lipids extracted from control virions and those exposed to phospholipase C. From the results shown in Table II, it is clear that while no hydrolysis of SPM or PS was observed following exposure of virions to phospholipase C, 95% of pyrene–PC and 35% of pyrene–PE were hydrolyzed by phospholipase C. Indeed, a similar observation was made with VSV grown in BHK cells in the absence of pyrene fatty acids, where ~95% of unlabeled PC and ~47% of PE were found to be accessible to phospholipase C hydrolysis (Patzer et al., 1978). Thus, it is evident from the data shown in Table II that pyrene–PC and pyrene–PE have asymmetrical distribution similar to that of unlabeled PC and PE. It is not possible to assess the distribution of pyrene–SPM and pyrene–PS, but they collectively represent only 5–8% of the total viral pyrene–lipid content. Moreover, the PS head group resists hydrolysis by phospholipase C (Patzer et al., 1978).

Pyrene Excimer Fluorescence in the Virion Membrane. Excimer formation of pyrene and its derivatives is a bimolecular reaction that serves as a very useful parameter to study lateral diffusion of membrane lipids (Galla & Hartmann, 1980; Lakowicz, 1981). In order to demonstrate the potential use of these pyrene lipids present in virion membrane, the fluorescence spectrum of PyC16 lipids in the VSV membrane was studied and compared with that of synthetic pyrene–C16PC. The emission spectrum of the pyrene-labeled virions obtained by exciting the viral suspension at 330 nm exhibited a broad excimer fluorescence at 470 nm and two sharp peaks at 385 and 410 nm due to monomer fluorescence (data not shown). This spectrum resembled very closely that of synthetic PyC16PC partitioned in POPC vesicles (5 mol %), and no peak representing the presence of free pyrene was observed in the spectrum. The excimer/monomer ratio (E/M) increased concurrently with increasing temperature due to the diffusion process (data not shown).

Spontaneous Transfer of Pyrene Lipids from Viral Membrane. It has been shown that phospholipids are transferred spontaneously between interacting vesicles with a half-time ranging from 2 to 720 h depending upon the physical state of the lipids in the bilayer and the lateral organization of the transferable species in the bilayer plane (Roseman & Thompson, 1980; Frank et al., 1983). Similar spontaneous transfer of phospholipids was also noted among lipoproteins (Massey et al., 1982). In contrast to the obligatory one-for-one

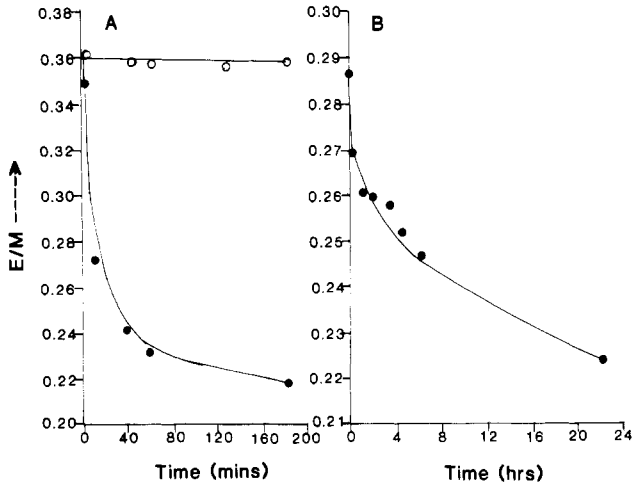

FIGURE 2: Spontaneous rates of transfer of pyrene lipids from virion membrane to acceptor serum lipoprotein molecules before (A) and after (B) removal of free fatty acids from VSV. (Panel A) VSV (200 μg) labeled with 16-(1-pyrenyl)hexadecanoic acid (PyC16) was added to 2 mL of PBS containing either no serum or 5% fetal calf serum at 37 °C, and the excimer/monomer fluorescence intensity ratio (E/M) was measured at various times thereafter. Virions treated with PBS alone (○); virions treated with 5% serum in PBS (●). (Panel B) VSV (500 μg) labeled with PyC16 fatty acid was incubated with 1 mL of 5% serum in PBS at 37 °C for 10 min. The virions were then purified by sucrose density gradient centrifugation as described under Materials and Methods. The fatty acid depleted virions (200 μg) were then added to 2 mL of PBS containing 5% fetal calf serum at 37 °C, and the excimer/monomer fluorescence intensity ratio (E/M) was measured at various times.

exchange reactions catalyzed by phospholipid exchange proteins (Wirtz, 1974), spontaneous transfer may lead to a net one-way transfer of lipids from one membrane to another.

It was important to determine whether pyrene lipids present in the VSV envelope can also undergo such spontaneous transfer to lipoproteins as has been reported for vesicles. Virions containing pyrene lipids in the membrane were incubated with PBS alone or with PBS containing 5% fetal calf serum (heat inactivated). The excimer/monomer fluorescence intensity ratio (E/M) at various time intervals after incubation at 37 °C was measured, and the results are shown in Figure 2A. The E/M ratio of VSV incubated with PBS alone showed no change with time, whereas the virions incubated with PBS containing 5% serum showed a very marked initial drop in the E/M ratio, which slowly decreased with time. The initial rapid drop in E/M ratio ($t_{1/2} \simeq 7$ min) was mainly due to the transfer of free fatty acids present in the viral membrane, which are known to transfer very rapidly (Doody et al., 1980). The kinetics of spontaneous transfer was reexamined after the free fatty acids were removed from the viral envelope. To this end, virions were first incubated at 37 °C with 5% serum in PBS for 10 min and then purified by centrifugation through a 0–40% sucrose density gradient (Barenholz et al., 1976). Such preincubation removed the free fatty acids from the virus membrane as determined by TLC of extracted viral lipids (data not shown). The pyrene fatty acid depleted virions were then incubated with 5% serum in PBS, and the E/M ratio was measured at different time intervals. As shown in Figure 2B, the E/M ratio decreased with time at a much slower rate ($t_{1/2} \geq 12$ h). These experiments show that interaction of virions with a lipid acceptor leads to spontaneous transfer of free pyrene fatty acids at a rapid rate and transfer of pyrene phospholipids at a much slower rate.

VSV–Vesicle Membrane Fusion Measured by Pyrene Excimerization. Vesicles containing VSV G protein (virosomes) have been shown to fuse with vesicles containing acidic phospholipids and only at low pH (Eidelmann et al., 1984). These observations prompted us to design experiments to study under more physiological conditions the kinetics of fusion with PS vesicles of intact VSV membrane containing endogenous pyrene phospholipids. The excimer/monomer ratio (E/M) provides a convenient method for continuous monitoring of the fusion reaction. As described above, there is a spontaneous rapid ($t_{1/2} \simeq 7$ min) transfer of free fatty acids to lipid receptors. Therefore, it was necessary to remove the free pyrene fatty acids from the VSV membrane by preincubation with serum lipoproteins in order to obtain accurate E/M ratio measurements of pyrene phospholipids, which transfer spontaneously to lipid acceptors at the much slower rate of $t_{1/2} \simeq$ 12 h. After interaction with 5% serum and purification by centrifugation in 0–45% sucrose gradients, the VS virions were found to be free of pyrene fatty acids by TLC (data not shown). These fatty acid depleted virions, but containing pyrene phospholipids, were tested for their reactivity with PS vesicles at pH 5.0 and pH 7.3. PS vesicles were chosen as receptors for interaction with the virion membrane because of several reports that PS is the putative cell-surface component for VSV adsorption. Schlegel et al. (1983) showed that PS vesicles markedly inhibited the high-affinity binding of VSV to Vero cells but PC vesicles did not. Eidelmann et al. (1984) confirmed this observation by finding that the fusion of G protein vesicles (virosomes) to receptor vesicles was dependent on the amount of PS in the receptor vesicles.

In our studies the fluorescence emission spectra of pyrene phospholipid labeled VSV in the presence of liposomes exhibited a typical broad excimer fluorescence at 470 nm and two sharp monomer peaks at fluorescence emissions of 410 and 385 nm. A similar spectral profile of pyrene phospholipids had been demonstrated repeatedly by Galla and Hartman (1980) and by Lakowicz (1981). During fusion of liposomes with VSV at pH 5.0, the excimer fluorescence decreased markedly with time, whereas the monomer emission intensity showed some increase. On the other hand, at neutral pH the spectra were unaffected with time of incubation of pyrene-labeled virions with PS liposomes (data not shown).

Figure 3 shows that pyrene phospholipid PyC16 in the VSV membrane undergoes a very sharp decline in E/M ratio on interaction with POPS SUV at pH 5.0 and 37 °C, indicating membrane fusion that was essentially complete by 10 min. By comparison, the pyrene E/M ratio showed very little change in interaction of VSV with POPS vesicles at pH 7.3 for a period as long as 60 min. The E/M ratio of the VSV pyrene phospholipid was not significantly affected at pH 5.0 in the absence of POPS receptor vesicles. The importance of the G protein in the fusion process was suggested by the finding that pyrene phospholipid PyC16 in liposomes formed by extracted VSV lipids in the absence of G (or any other) protein exhibited little alteration in the E/M ratio (Figure 3). Moreover, the fusion reaction at pH 5.0 with POPS vesicles was markedly reduced when VS virions had been rendered free of glycoprotein spikes by exposure to thermolysin (data not shown). These studies strongly suggest, therefore, the VSV membrane undergoes rapid fusion with PS receptor vesicle membrane at pH 5.0, but not at neutral pH, and this reaction is modulated by the G protein in a specific conformational configuration. It is also apparent that excimerization of an endogenous pyrene phospholipid probe provides a useful and biologically natural method for monitoring fusion of a viral membrane with a model receptor membrane.

Comparative Fusion Studies with an Exogenous Fluorophore. It was of interest to compare the membrane fusion

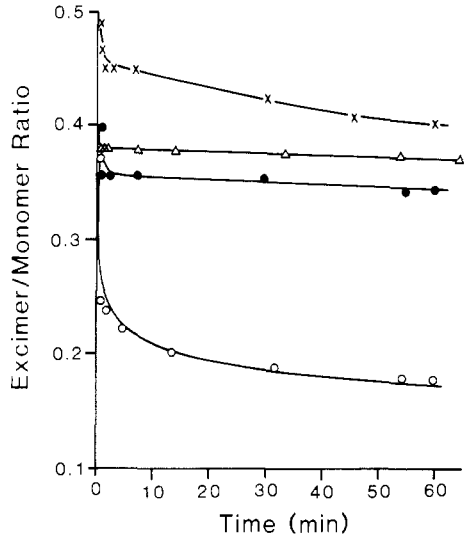

FIGURE 3: Fusion of intact VSV or VSV lipid vesicles with 1-palmitoyl-2-oleoylphosphatidylserine (POPS) small unilamellar vesicles (SUV) at pH 5.0 or pH 7.3 as measured by excimer/monomer fluorescence intensity ratio. VSV (5 mg) labeled metabolically with PyC16 fatty acid was incubated with 5 mL of 5% fetal calf serum (heat inactivated) in PBS at 37 °C for 15 min to remove free pyrene fatty acids. The virions were then purified by 0–40% sucrose gradient centrifugation as described elsewhere (Barenholz et al., 1976). Fatty acid depleted virus (100 μg) was then added to 3 mL of 10 mM citrate buffer, pH 5.0 or pH 7.3 at 37 °C, and the virus–vesicle fusion was initiated by adding 150 nmol of POPS vesicles. The excimer/monomer fluorescence ratio intensity (E/M) was determined over a period of 1 h by exciting the sample at 330 nm; emission was measured at 385 and 470 nm for monomer and excimer, respectively. VSV lipid vesicles were prepared by extracting the lipids from pyrene-labeled virions (200 μg) by the method of Folch et al. (1957), and the protein-free vesicles were prepared by sonicating the VSV lipid suspension in 10 mM Tricine, pH 7.5. Fusion of viral lipid vesicles with POPS vesicles were performed as described above. VSV alone at pH 5.0 (△); VSV with POPS vesicles at pH 5.0 (○); VSV with POPS vesicles at pH 7.3 (●); VSV lipid vesicles with POPS vesicles at pH 5.0 (×).

kinetics determined by energy exchange of the endogenous pyrene phospholipid probe with that of a well-studied exogenous probe, octadecyl Rhodamine B. This Rhodamine fluorophore readily partitions into biological membranes at self-quenching concentrations; upon fusion of the quenched fluorophore-containing membrane with a receptor membrane devoid of the probe, the surface density of the Rhodamine fluorophore decreases, resulting in a marked increase in fluorescence intensity (Hoekstra et al., 1984). This dequenching of the exogenous probe can be monitored continuously to measure the rate of membrane fusion.

VSV was labeled with a quenching concentration of octadecyl Rhodamine B by incubating purified virions with the fluorophore for 60 min at 37 °C. To labeled virions in citrate buffer at pH 5.0 or pH 7.3 were added sonicated POPS vesicles, and the fluorescence intensity at 560 nm was monitored over a period of 45 min. Figure 4 reveals very rapid dequenching of the Rhodamine probe in virions incubated at 37 °C with POPS vesicles at pH 5.0. In contrast, only a minimal amount of the Rhodamine fluorophore was dequenched on incubation with POPS vesicles at pH 7.3. In both cases, plateaus of unquenched fluorophores were reached within 3 min. These data with the exogenous Rhodamine probe agree well with results of the VSV membrane fusion experiments with the endogenous pyrene phospholipid energy exchange. Clearly, in each case, fusion of VSV membrane with receptor POPS vesicles is pH dependent. At this low pH, fusion occurs very rapidly with a short half-time ($t_{1/2} \simeq 2$ min) and is virtually completed within 10 min.

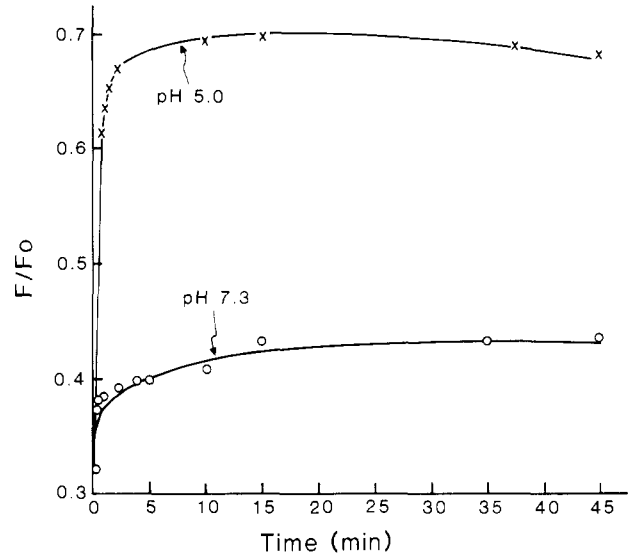

FIGURE 4: Fusion of VSV with POPS vesicles probed by dequenching of octadecyl Rhodamine B chloride (R_{18}) as measured by the ratio of quenched fluorescence intensity (F) to completely unquenched fluorescence intensity (F_0). VSV (1 mg) was incubated with octadecyl Rhodamine B chloride (1:100 probe to lipid molar ratio) in PBS at 37 °C for 45 min. (The R_{18} probe had been added to the viral suspension in ethanol so that the final ethanol concentration in solution was less than 0.5%.) The free probe molecules were removed by pelleting the virions in the SW50.1 rotor at 25 000 rpm for 60 min through a 5% glycerol pad. The R_{18}-labeled virions (100 μg) were then added to 3 mL of 10 mM citrate buffer, pH 5.0 or pH 7.3 at 37 °C, and the fusion was initiated by adding 150 nmol of POPS vesicles. The emission intensity (F) was measured at 590 nm after the probe was excited at 560 nm. The total unquenched fluorescence (F_0) was obtained by complete solubilization of R_{18}-labeled virions with 1% Triton X-100.

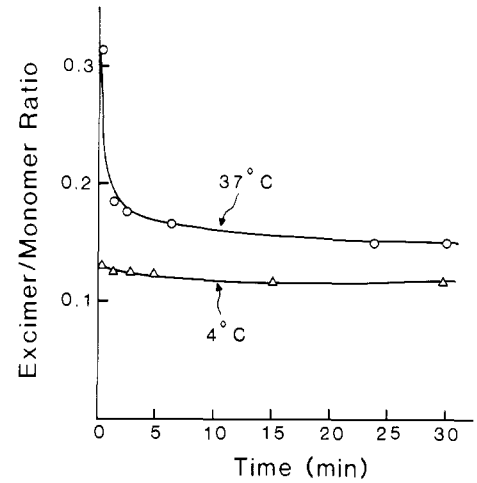

FIGURE 5: Temperature dependence of VSV membrane fusion at pH 5.0 with POPS vesicles. VS virions biologically labeled with PyC16 fatty acid were depleted of free fatty acid as described in the legend of Figure 3. Fatty acid depleted virions (100 μg) were incubated with 150 nmol of POPS vesicles at 4 °C for 30 min. The suspension of virions was then added to 3 mL of 10 mM citrate buffer, pH 5.0 at 37 °C (○), and the E/M ratio was measured at intervals for 30 min as described in the legend of Figure 3. Fusion of VSV with POPS vesicles at 4 °C (△) was monitored by adding fatty acid depleted VSV (100 μg) to 3 mL of 10 mM citrate, pH 5.0 at 4 °C, and the fusion was initiated by adding 150 nmol of POPS vesicles.

Temperature-Dependent Fusion of VSV and POPS Membranes. The foregoing fusion experiments were performed at 37 °C. To gain some insight into the energy dependence of the fusion reaction, fluorescence transfer of pyrene phospholipid in the VSV membrane was compared at 4 and 37 °C during interaction with POPS receptor vesicles at pH 5.0.

Figure 5 reveals that the E/M ratio of pyrene phospholipid PyC16 underwent no significant change when incubated with POPS vesicles at pH 5.0 at 4 °C for 30 min. When VSV labeled with somewhat greater amounts of pyrene phospholipid was preincubated at pH 5.0 with POPS vesicles at 4 °C and then warmed to 37 °C, a rapid decline in the E/M ratio was observed, reaching a plateau at ~5 min. It seems likely that the pyrene phospholipid labeled virions attached to POPS vesicles at 4 °C but the fusion event is triggered by increase in the reaction temperature. In all likelihood, adsorption of VSV to the surface of POPS vesicles is electrostatic in nature and has a low energy requirement, whereas fusion is highly energy dependent.

Effect of Antibodies to G Protein on VSV Membrane Fusion. The evidence is quite conclusive that the G protein is the VSV site for host–membrane recognition as well as being the fusion factor (Pastan & Willingham, 1983; Schlegel et al., 1982; Eidelmann et al., 1984). Removal of most of the G protein by exposure of virions to proteases greatly reduces viral infectivity (Bishop et al., 1975), and exposure of intact VSV to certain G-specific monoclonal antibodies, but not others, neutralizes viral infectivity (Volk et al., 1982). With these observations in mind, we set out to determine to what extent the intact G protein on the VSV surface was responsible for pyrene phospholipid energy transfer on fusion of VSV and POPS membranes. We tested the effect of monoclonal antibodies on the rate and extent of decline in the E/M ratio of pyrene phospholipids following VSV interaction with POPS vesicles.

We had previously prepared mouse hybridomas secreting immunoglobulins that could be assigned to 11 separate antigenic determinants (epitopes) on the G protein; monoclonal antibodies (mAb) to four epitopes neutralized the infectivity of VSV, but mAbs to the other seven epitopes did not (Volk et al., 1982). We tested two of these monoclonal antibodies, mAb6, which neutralizes VSV infectivity, and mAb17, which does not, for their capacity to inhibit the virion fusion activity. VSV was incubated with mAb6 or mAb17 (150 μg of IgG of each) for 60 min at room temperature, and these virions as well as control virions each containing pyrene phospholipids were incubated with POPS vesicles at pH 5.0 and 37 °C. As shown in Figure 6, both mAb6 and mAb17 (to a somewhat lesser extent) inhibited the reduction in E/M ratio of PyC16 phospholipid exhibited by control VSV on interaction at pH 5.0 with POPS vesicles. Since these two monoclonal antibodies to two separate G protein antigenic determinants both had a dramatic effect on the VSV fusion reaction, this experiment does not account for the markedly different capacities of these two monoclonal antibodies to neutralize viral infectivity.

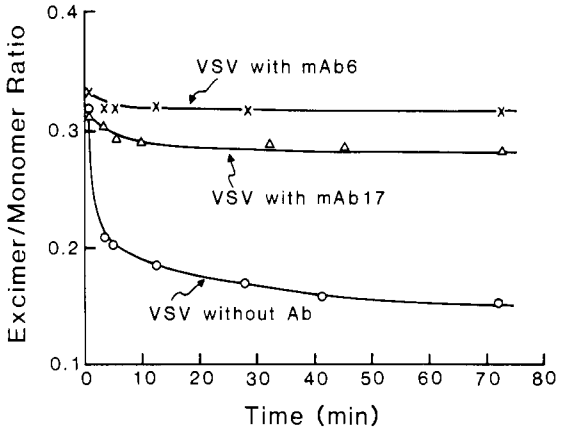

FIGURE 6: Effect of monoclonal antibodies to G protein on the fusion of VSV with POPS vesicles. Virions labeled with PyC16 were depleted of free fatty acids as described in the legend of Figure 3. Fatty acid depleted virions (100 μg) were then incubated with purified IgG (150 μg) of neutralizing (mAb6) or nonneutralizing (mAb17) monoclonal antibody to G protein at room temperature for 60 min. The virion–antibody mixture was then added to 3 mL of 10 mM citrate buffer (pH 5.0), and the fusion reaction was performed at 37 °C by adding 150 nmol of POPS vesicles. Control VSV without antibody (○); VSV pretreated with mAb6 (×); VSV pretreated with mAb17 (△).

Discussion

Pyrene fatty acids of nearly all chain lengths (C_9–C_{16}) were found to be incorporated into phospholipids of the VSV membrane. An increase in the polarity of these pyrene fatty acids by addition of a keto group in the pyrene moiety significantly reduced its incorporation into lipids of the viral membrane. It has been suggested that pyrene keto fatty acids are not transported efficiently across cell membranes because the hydrophilic keto group presumably inhibits incorporation into the outer leaflet of the membrane (Morand et al., 1982). Assuming that every fluorescent phospholipid contains only one fluorescent fatty acyl chain, we calculate that nearly 8 mol % of viral phospholipids can be labeled with the fluorescent probe when pyrenylhexadecanoic acid was used as a biological label for the host cell lipids. Viral phospholipids represented 85–90% of the fluorescent fatty acid incorporated by the viral membrane. Pyrene lipids in the membrane of VSV had no effect on viral infectivity.

The pyrene–PC and pyrene–PE present in the intact VSV membrane showed different susceptibility to phospholipase C hydrolysis, thus suggesting two different pools ostensibily representing the inner and outer monolayers of the viral membrane. Most of the pyrene–PC in the VSV membrane was accessible to phospholipase C hydrolysis, while only 35% of the pyrene–PE was hydrolyzed under the same conditions. Similar observations were noted for unlabeled PC and PE when VSV grown in BHK cells was treated with phospholipase C (Patzer et al., 1978). Therefore, bulky pyrene phospholipids apparently did not affect the distribution of PC and PE in the two pools presumably representing the outer and inner monolayer of the VSV membrane. No definite conclusions about the distribution of pyrene–SPM and pyrene–PS could be reached because the level of incorporation of pyrene fatty acids in these lipid classes was very low in VSV membrane. The pyrene lipids in the virion membrane were found to transfer spontaneously to lipoprotein molecules in heat-inactivated serum. However, pyrene phospholipids were found to transfer at a much slower rate than did free fatty acids. Similar rates of spontaneous transfer of phospholipids were also demonstrated on interaction between phospholipid vesicles and serum lipoproteins (Roseman & Thompson, 1980; Frank et al., 1983; Massey et al., 1982).

Enveloped animal viruses enter host cells and establish infection by a process involving fusion of the virion membrane with a cellular membrane. In the case of Sendai paramyxovirus the fusion events occur in a neutral pH environment at the plasma membrane of the host cell (Choppin & Compans, 1975). For many other enveloped viruses, such as influenza, VSV, and Semiliki Forest virus, the mode of interaction of virions with the host cell is quite complicated. Evidence presented in recent years indicates that entry of these viruses occurs by means of coated pits translocated through the membrane to form intracellular coated vesicles. In the low pH environment of these endosomes, the virion envelope fuses with the limiting membrane of the endosome, releasing the viral nucleocapsid into the cytoplasm (Matlin et al., 1982; Schlegel et al., 1982; White et al., 1983; Pastan & Willingham, 1983). For VSV such low pH-dependent fusion activity has

also been demonstrated in vitro with cultured cells, erythrocytes, and liposomes as targets (Yamada & Ohnishi, 1986; Eidelmann et al., 1984).

Pyrene lipid labeled virions appear to provide useful probes to study the kinetics of host–virus interaction because the fluorescent virions are biologically labeled and retain infectivity and the pyrene lipids undergo a rapid change in excimerization on membrane interaction. Moreover, the measurement of pyrene excimerization is consistent and probably quite accurate as well as being relatively simple. Since the half-time of spontaneous transfer of phospholipids from virions is very slow, it is possible to follow the kinetics of fusion of enveloped virions with various membrane targets since such a fusion process rapidly triggers the excimer fluorescence event to a marked extent.

It has been repeatedly demonstrated that the G protein plays a paramount role in VSV membrane fusion (Florkiewicz & Rose, 1984; Woodgett & Rose, 1986; Schlegel & Wade, 1984, 1985). Florkiewicz and Rose (1984) have shown that a stable cell line transformed by a VSV G protein DNA expression vector undergoes fusion and forms polykaryocytes at low pH. Synthetic peptides corresponding to the N-terminal domain of the VSV G protein are capable of lysing erythrocytes at pH 5.0, a reaction equivalent to the fusion process (Schlegel & Wade, 1984, 1985). In the present study with pyrene phospholipid labeled virions, it has been demonstrated that the G protein plays an important role in mediating fusion of VSV with model membranes. The fusogenic activity of the G protein is implicated by the finding that viral lipid vesicles free of viral proteins did not undergo fusion with PS vesicles at pH 5.0. Furthermore, monoclonal antibodies to G protein of both neutralizing and nonneutralizing types inhibited virus–liposome fusion markedly. However, this effect of antibody on VSV membrane fusion should be interpreted with caution since binding of antibodies to VSV spikes might also induce aggregation of the virions, thus reducing fusion sterically.

Registry No. POPS, 79980-16-8.

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