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pH-Dependent Lysis of Liposomes by Adenovirus

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ABSTRACT: Purified adenovirus induced a dose-dependent release of the water-soluble markers calcein and carboxyfluorescein from liposomes. Marker release was strongly dependent on pH, and at temperatures below 5 °C, the rate of release showed an optimum at a pH of about 6. This pH dependence parallels disruption of endocytic vesicles by adenovirus and the permeabilization that adenovirus induces on the cell surface. There did not seem to be a striking dependence on the lipid composition of the liposomes. Electron microscopy using a negative stain shows liposomes bound to adenovirus. In some cases, the liposomes were still intact, but many liposomes, which were attached to the vertices of the virus, appeared lysed. These data support the notion that adenovirus, which enters the host cell by receptor-mediated endocytosis, gains access to the cytoplasm by a subsequent pH-dependent disruption of the membrane of the endocytic vesicle.

Adenovirus is a nonenveloped virus whose nucleocapsid finds its way to the cytoplasm of the host cell by receptor-mediated endocytosis, followed by disruption of the the membrane of the endocytic vesicle (Dales, 1973; FitzGerald et al., 1983). This disruption is pH dependent and is abolished by treating cells with compounds that raise the pH of the endocytic vesicle (Seth et al., 1984a). The disrupting activity can be shown to take place directly on the plasma membrane, if cells with adenovirus attached to their surface receptors are placed at a decreased pH (Seth et al., 1984c).

In order to examine the mechanism of this membrane disruption, we studied the interaction of adenovirus with liposomes. In the present study, we show that purified adenovirus induces a rapid, pH-dependent release of the water-soluble markers calcein and carboxyfluorescein from liposomes. Electron micrographs suggest attachment of liposomes to the

vertices of the virus, which might provide the hydrophobic sites required for membrane disruption.

Experimental Procedures

Preparation of Liposomes. Phospholipids were obtained from Avanti Biochemicals (Birmingham, AL); cholesterol was from Calbiochem-Behring (San Diego, CA). Small unilamellar vesicles (SUV, approximately 25 nm in diameter) containing calcein (GFS Chemicals, Columbus, OH) were prepared as previously described (Blumenthal et al., 1984) by sonication in the presence of 100 mM fluorophore, pH 7.4, followed by elution from a Sephadex G25 PD-10 column (Pharmacia, Piscataway, NJ) into 145 mM NaCl and 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4. Dipalmitoylphosphatidylcholine (DPPC) liposomes were made by incubation of DPPC SUV for 2 days at 21 °C; this resulted in fusion of the SUV into larger vesicles (about 70 nm in diameter) according to Schullery et al. (1980). Detergent-dialyzed vesicles were prepared by dissolving lipid in a 100 mM calcein solution, pH 7.4, containing 30 mM octyl glucoside (Calbiochem, La Jolla, CA). The suspension was dialyzed in a dialysis bag against small volumes of a 100 mM calcein solution according to the procedure of Eidelman et al. (1984). This slow removal of detergent from lipid results in the formation of lipid vesicles with a homogeneous size distribution. After the octyl glucoside concentration had reached about 15 mM, the suspension was eluted on a PD-10 column and dialzyed overnight against 500 mL of buffer to remove residual octyl glucoside. The resulting liposomes were homogeneous in their size distribution, unilamellar, and about 70 nm in diameter as determined by sizing column chromatography, quasi-elastic light scattering using a Coulter Model N4 submicrometer particle analyzer, and electron microscopy using a negative stain.

Fluorescent Marker Release. The assay for release makes use of the self-quenching properties of a highly water-soluble fluorescent dye. The method was originally developed for the dye carboxyfluorescein (Weinstein et al., 1977), but calcein (Allen, 1984) was used in most of these experiments since its fluorescence is fairly constant in the pH range 5–8 in contrast to carboxyfluorescein whose fluorescence decreases steeply below pH 7. However, since calcein fluorescence is sensitive to the presence of divalent ions, those experiments were done in the presence of a chelator [ethylenediaminetetraacetic acid (EDTA) or citrate]. The fluorescence intensity of the dye encapsulated in vesicles at 100 mM concentration is about 5% of that obtained when the dye is released and diluted into the medium. The percentage of dye release by various combinations of adenovirus and pH was determined by measuring fluorescence before and after addition of the detergent $C_{12}E_9$ (Calbiochem, La Jolla, CA). Recordings were done at an excitation wavelength of 470 nm and an emission wavelength of 520 nm using a Perkin-Elmer Model MPF 44B spectrofluorometer. An aliquot of 1–50 μL of adenovirus suspension was added to 2.5 mL of a vesicle suspension (0.1 μM in lipid) in buffer in a 1 × 1 cm cuvette and mixed.

Adenovirus. Adenovirus type 2 was grown and propagated in KB cells in suspension cultures. The virus was purified on CsCl gradients as described previously (FitzGerald et al., 1983), UV-inactivated by exposure to a 15-W germicidal lamp [G15T8 (GE)] for 15 min, and stored in a tris(hydroxymethyl)aminomethane (Tris)–saline–30% glycerol buffer at −70 °C.

Binding Assay. Adenovirus (50 μg/mL) was incubated with SUV made of egg PC containing 0.1% [^{14}C]DPPC for 10 min at either 23 or 4 °C in 1 mL of buffer containing 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 25 mM HEPES, and 100 mm NaCl adjusted to various pH values. The samples were centrifuged at 90000g_{av} in a Beckman type 40 rotor at either 23 or 4 °C, and the radioactivity in the supernatant was counted. All the virus spun down under those conditions, but no liposomes spun down in the absence of virus. The results are expressed as percent bound which equals $(1 - \text{cpm}_{\text{supernatant}}/\text{cpm}_{\text{total}}) \times 100$.

Electron Microscopy. Electron microscopy using a negative stain requires removal of glycerol from the adenovirus preparation. Forty micrograms of adenovirus was incubated for 15 min with SUV (4 μg of lipid) in 200 μL of 50 mM sodium citrate buffer, pH 6.1, containing 30% glycerol. The suspension was diluted into 2 mL of HEPES buffer, pH 7.4 resulting in 70% release of contents. The suspension was then concentrated in a negative-pressure micro protein dialysis concentrator (ProDiCon, Beaverton, OR) for 12 h at 4 °C. After dialysis, the mixture was spread on a glow-discharged carbon-coated Formvar film grid, blotted, and while still wet overlaid with 2% sodium phosphotungstate, pH 7.2. The grid was examined with a Philips 400T electron microscope at 40 kV.

Results

pH-Dependent Adenovirus-Mediated Calcein Release from Liposomes. Figure 1 shows adenovirus-mediated release of calcein from liposomes as a function of pH. These liposomes, formed by detergent dialysis, were unilamellar and about 70 nm in diameter. We use the term liposome to include small unilamellar vesicles (SUV) and larger unilamellar vesicles formed by detergent dialysis. These experiments were done at 2 °C for reasons which will become clear later. There appears to be an initial lag, perhaps related to the time it takes to form a "lytic complex", followed by a steep rise (Figure 1A). Similar kinetics have been observed in studies on release from liposomes mediated by cytolysin (Blumenthal et al., 1984). The rate of fluorescence increase in the steep portion can be related to the number of lytic complexes formed. In Figure 1B, we have plotted this rate as a function of pH. The rate of adenovirus-mediated release shows a pH dependence with an optimum at a pH of about 6. This pH dependence is very similar to the pattern of adenovirus-mediated permeability changes seen in cells (Seth et al., 1984c).

After partial adenovirus-induced release from liposomes, subsequent addition of adenovirus did not induce further release of the remaining marker (data not shown). Adenovirus-mediated release did not depend on the presence of divalent cations. Curves similar to those shown in Figure 1 were obtained in the presence of 5 mM Ca^{2+} or 5 mM EDTA (data not shown). Those experiments were done with carboxyfluorescein-containing vesicles, because calcein fluorescence is sensitive to Ca^{2+}. Release was not sensitive to salt concentrations up to 0.2 M NaCl. However, above 0.2 M NaCl, release was inhibited. Likewise, 30% glycerol, 0.6 M sucrose, bovine serum albumin (1 mg/mL), or whole serum also inhibited release. Sendai virus, vesicular stomatitis virus, or bacteriophage λ added to those liposomes at higher virus concentrations and at a variety of pHs did not induce any release of contents (data not shown).

Variation of Liposome Lipid Composition. Vesicles with different lipid compositions were tested as targets for adenovirus-induced calcein release. The liposomes used in the experiment shown in Figure 1 were made of egg phosphatidylcholine (PC) alone. Figure 2 shows how this rate compares with that for liposomes with a variety of different lipid compositions at pH 6.1 and 2 °C.

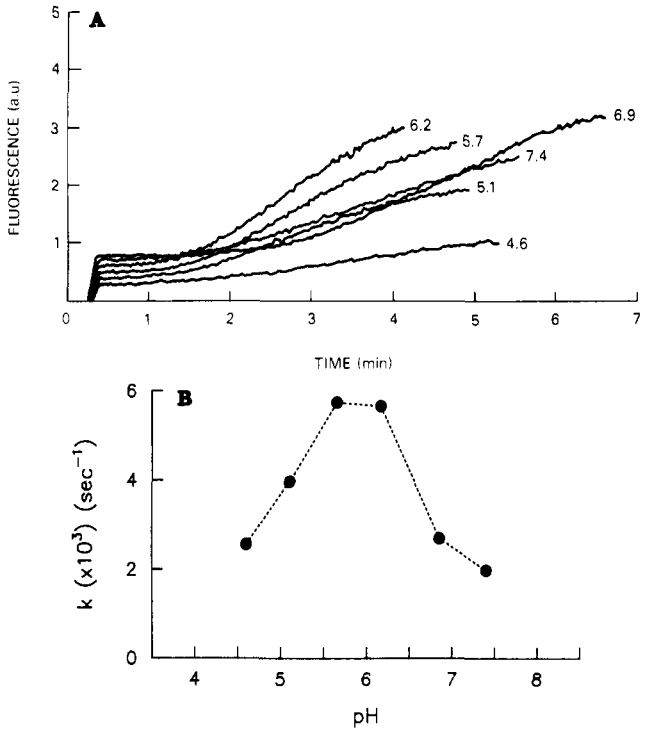

FIGURE 1: Release of calcein from liposomes induced by adenovirus. The liposomes were composed of egg PC, formed by detergent dialysis, and were about 70 nm in diameter. (A) Time course; (B) rate of release of calcein from liposomes at 2 °C; rate constant calculated from $(\Delta F/\Delta t)/(F_t - F_0)$ where ΔF is the difference between two fluorescence values on the steep portion of the curves in Figure 1A at time intervals Δt, F_0 is the background fluorescence, and F_t is the total fluorescence released by $C_{12}E_9$. Adenovirus (protein) concentration was 2.5 μg/mL, and lipid concentration was 0.1 μg/mL. These experiments were carried out at 2 °C in 50 mM sodium citrate adjusted to the appropriate pH.

Figure 2 shows that adenovirus mediated virtually no release from liposomes made of dipalmitoylphosphatidylcholine (DPPC), whose lipids are in the solid phase at this temperature. Inclusion of 40% cholesterol into egg PC liposomes markedly enhanced the initial rate of release, but the extent was about the same as that for PC. Replacement of 50% of the PC with the negatively charged phospholipid phosphatidylserine (PS) enhanced both the rate and extent of release. In these experiments, it was very important to control for liposome size. The liposomes were about the same size (70 nm in diameter) as determined by a variety of techniques (see Experimental Procedures).

Liposomes made of a partially purified soybean lecithin extract, which contain considerable amounts of phosphatidylethanolamine and phosphatidylinositol, were not significantly different from PC liposomes; neither did inclusion of gangliosides into the lipid mixture alter the release properties (data not shown). We can conclude from these experiments that other than a failure to release contents from the tightly packed gel-phase DPPC vesicles there did not seem to be a marked lipid selectivity for adenovirus-mediated permeabilization of membranes.

Dependence on Adenovirus to Lipid Ratio. The dependence of the rate of release at low temperature and pH showed a complex pattern (Figure 3A). At an adenovirus/lipid ratio of 10, there was a long (2–3 min) delay, which was followed by a steeper rize until a maximal release of about 90% was reached. With increasing adenovirus/lipid ratios, this delay became shorter, and the rise steeper. However, above an adenovirus/lipid ratio of 100, the slope of the steep portion decreased again, indicating a decrease in the rate of release.

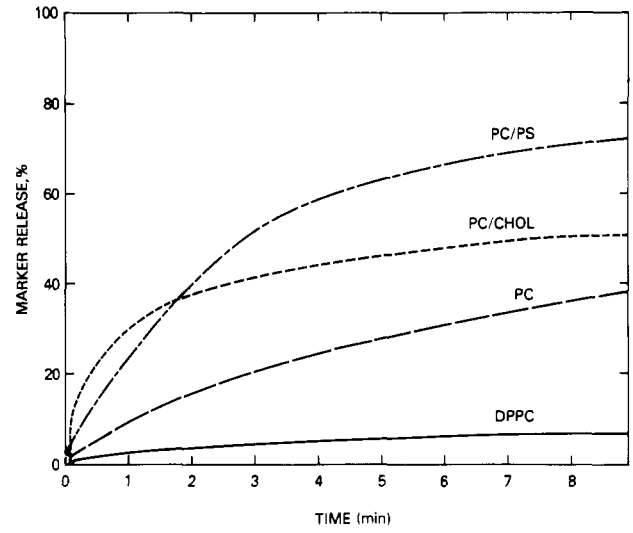

FIGURE 2: Dependence of adenovirus-induced calcein release on liposome composition. PC:PS (1:1) and PC:cholesterol (1.5:1) liposomes were formed by detergent dialysis, and DPPC liposomes were formed by fusion of SUV. The liposomes were 70 nm in diameter. This experiment was performed in 50 mM sodium citrate buffer, pH 6.1 at 2 °C. The final adenovirus and lipid concentrations were 2.5 and 0.1 μg/mL, respectively.

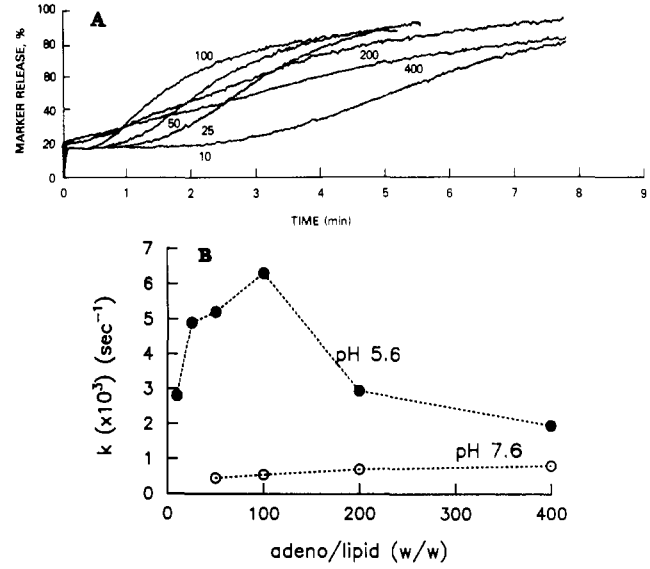

FIGURE 3: Dependence of the kinetics of adenovirus-induced calcein release on concentration. Lipid (egg PC) concentration was constant at 0.1 μg/mL; virus concentration was varied. Experiments were done in citrate buffer at 2 °C. (A) Complete release curves at pH 5.6. (B) Rate constant for release calculated as in the legend to Figure 1; (●) pH 5.6; (○) pH 7.6.

Close to maximal release was reached after about 10 min at all adenovirus/liposome ratios. In Figure 3B, we have plotted the rate of release as calculated from the steep portion as a function of adenovirus/lipid ratio. It shows that a maximum rate is reached at an adenovirus/lipid ratio of 100 and that the rate subsequently decreased at higher adenovirus/lipid ratios. Figure 3B also shows that at pH 7.5 their was little release of calcein at all the adenovirus concentrations used.

Our interpretation of this complex curve at pH 5.6 is that adenovirus self-aggregates at higher concentrations. By measuring the optical density of adenovirus in the absence of glycerol or liposomes, we have observed that aggregation of virus increases as the pH is lowered from 7.4 to 5.0 (data not shown). At low temperature and low virus concentration, there is presumably little aggregation. However, as shown in Figure 3, at higher adenovirus concentrations where aggregation can

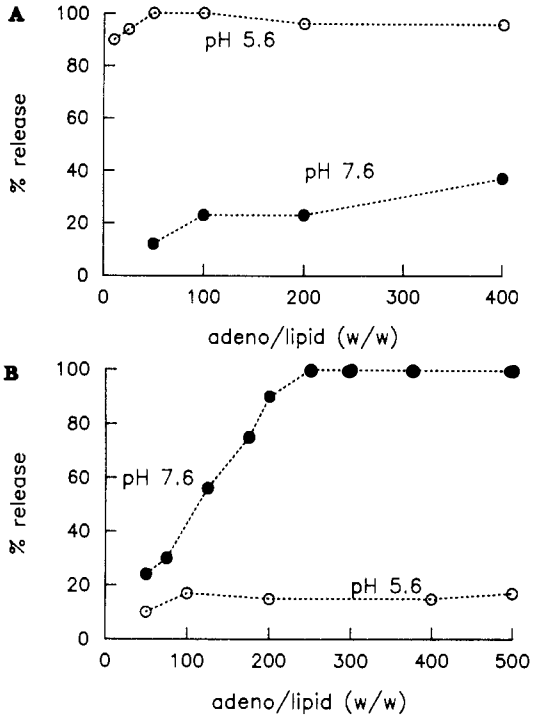

FIGURE 4: Dependence of the extent of adenovirus-induced calcein release on virus concentration. (A) Conditions as in the legend to Figure 3. Percentage release was calculated from $[(F_s - F_0)/(F_t - F_0)] \times 100$, where F_s is the steady-state fluorescence reached after 10 min; F_0 and F_t are defined as in the legend to Figure 1. (B) As in (A) but the temperature was 23 °C. In both (A) and (B), closed circles represent pH 7.6 and open circles are pH 5.6.

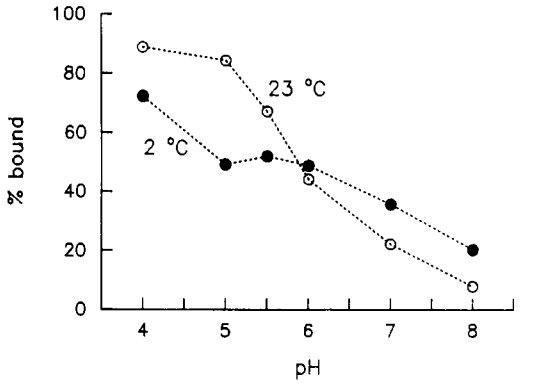

FIGURE 5: Binding of liposomes to adenovirus. Egg PC liposomes (2 μg/mL) containing 0.1% [^{14}C]DPPC were incubated with adenovirus (50 μg/mL) for 10 min at 23 °C (○) and 2 °C (●). Subsequently, the mixture was spun at 90000g_{av} for 10 min, and supernatants were counted. All the adenovirus pelleted under those conditions. Liposomes by themselves did not pellet under those conditions. % bound = $(1 - \text{cpm}_{\text{supernatant}}/\text{cpm}_{\text{total}}) \times 100$.

take place release was inhibited.

Temperature Dependence of Release. In Figure 4 we have plotted the extent of release as a function of the adenovirus/lipid ratio at two pHs and at two temperatures. At 2 °C, close to maximal release was obtained at pH 5.6, at an adenovirus/lipid ratio of 10, whereas release was significantly less at pH 7.6 even at an adenovirus/lipid ratio of 400 (Figure 4A). On the other hand, at room temperature there was very little release at pH 5.6 even at adenovirus/lipid ratios of 500 (Figure 4B). As shown in Figure 4B, it was possible to obtain release at pH 7.6. However, the adenovirus/lipid ratios required to induce maximal release were much higher (above 250) than those required to induce full release at 2 °C and pH 5.6. We attribute the failure to induce release at pH 5.6 and room temperature to virus self-aggregation, which is

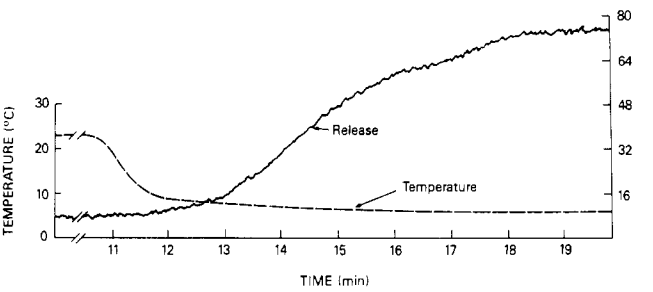

FIGURE 6: Release of calcein from liposomes induced by adenovirus at pH 5.5 by lowering the temperature. Egg PC liposomes (0.1 μg/mL) were incubated at 23 °C with adenovirus (2.5 μg/mL) in citrate buffer, pH 6.1. The dashed line shows the change in temperature; the solid line shows release of contents.

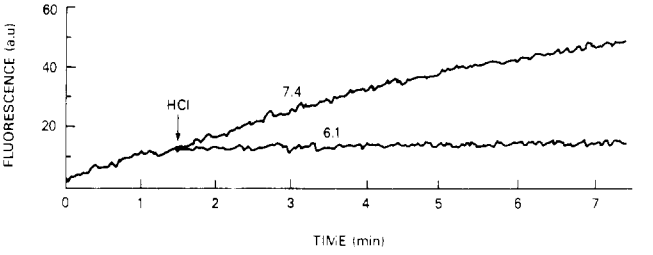

FIGURE 7: Effect of lowering the pH on adenovirus-mediated release at room temperature. Egg PC liposomes (0.1 μg/mL) were incubated with adenovirus (20 μg/mL) at pH 7.4, 23 °C. At the arrow, 5 μL of 0.1 N HCl was added, lowering the pH to 6.1.

significantly higher at 23 °C than at the low temperature.

As shown in Figure 5, liposomes bind to the adenovirus in a pH-dependent fashion, both at room temperature and at 2 °C. Presumably, self-aggregated virus can bind to liposomes without causing release.

Reversibility of Liposome–Virus Interaction. The inhibitory interaction at room temperature and low pH was not irreversible. This is demonstrated in Figure 6. Incubation of liposomes with adenovirus at room temperature, pH 5.6, caused virtually no release for 10 min. When the temperature was subsequently lowered to about 5 °C, release was initiated. Likewise, as shown in Figure 7, adenovirus-induced release at room temperature, pH 7.6, can be blocked by lowering the pH to 6.1.

If we assume that self-aggregation of virus at room temperature and low pH blocks release, these data indicate that this self-aggregation can be reversed by lowering the temperature or increasing the pH. This is consistent with our observation that aggregation of virus, monitored by optical density, as a function of pH is reversible (data not shown).

pH-Dependent Release at Room Temperature by Preincubation with Glycerol. We had only seen significant adenovirus-mediated release in the pH range expected from studies with cells when we cooled the assay medium down to 2 °C. We attributed our failure to observe release at room temperature in this pH range to self-aggregation of virus. Adenovirus is stored in 30% glycerol to prevent self-aggregation. This concentration of glycerol also prevents adenovirus-mediated marker release from liposomes (data not shown), presumably by diminishing hydrophobic interactions between virus and lipid. However, rapid dilution into glycerol-free buffer after preincubation with glycerol led to an instantaneous release. Figure 8 shows the extent of release as a function of the adenovirus/lipid ratio after preincubation of virus and liposome with glycerol at two pHs. At a sufficiently high adenovirus to vesicle ratio, essentially all the encapsulated calcein was released within 1 min. At lower adenovirus to lipid ratios, the extent of release was less. The figure shows that

Table I: Internal Fluorescence after Partial Adenovirus-Induced Release from Liposomes

adenovirus/lipid ratio	fraction of unreleased calcein[a]	rel fluorescence/units of phospholipid (predicted all or none)[b]	rel fluorescence/ units of phospholipid (predicted graded)[c]	rel fluorescence/ units of phospholipid (exptl result)[d]
0	1	14	14	14
3	0.76	11	16	15
29	0.33	5	20	28

[a] Calculated from $(F_t - F_s)/(F_t - F_0)$ where F_0 is the background calcein fluorescence, F_s is the fluorescence at steady state after addition of adenovirus, and F_t is the fluorescence after adding $C_{12}E_9$; egg PC SUV were used in these experiments. [b] Assumes some vesicles lost all calcein and others lost none; (column 2) × 14 (the measured value before leakage). [c] Assumes all vesicles are equal and release the same amount of calcein, giving rise to concentrations of calcein in vesicles of 100, 76, and 33 mM, respectively. Values obtained from the calcein fluorescence quenching curve (Allen, 1984). [d] Determined after removal of released calcein by using a PD-10 column.

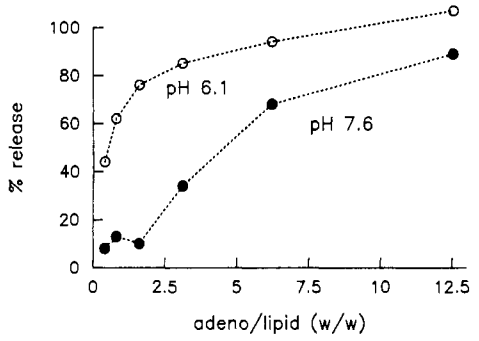

FIGURE 8: Effect of preincubation with glycerol on adenovirus-mediated release. Egg PC liposomes (0.1 μg/mL) were preincubated for 15 min at 23 °C with adenovirus at different concentrations at pH 6.1 (○) or pH 7.6 (●) in 200 μL of citrate buffer containing 30% glycerol. Subsequently, the mixture was diluted into 2 mL of NaCl–HEPES, pH 7.6, without glycerol, and fluorescence was measured at 23 °C before and after addition of detergent.

at pH 6.1 the percent released was markedly greater than at pH 7.6 at low adenovirus/lipid ratios. Both preincubation and dilution were done at 23 °C; a similar extent of release was obtained whether the pH of the release medium was 7.4 or 6.1.

The effect was not specific for glycerol; preincubation with 0.6 M sucrose (during which there was no release) followed by dilution in a sucrose-free medium produced a similar dose–response curve to that shown in Figure 8. It could be argued that the glycerol effect is osmotic: glycerol permeates into the vesicle during the preincubation time, and dilution into the glycerol-free medium causes an instantaneous osmotic gradient between the vesicle lumen and the medium. However, these vesicles are impermeable to sucrose, which has the same effect. Moreover, the dilution experiment done with small unilamellar vesicles, which are osmotically insensitive, shows similar results.

Mechanism of Marker Release from Liposomes. The self-quenching properties of calcein can be used to design experiments to test whether adenovirus-induced calcein release was a graded or on all or none response from individual liposomes; i.e., when half the calcein was released, had all the vesicles lost half their calcein or had half the vesicles lost all their calcein? This experiment is possible since the efficiency of calcein fluorescence varies with its local concentration; it is "self-quenched" at high concentrations such as the original calcein concentration inside the liposomes (Allen, 1984). The level of fluorescence of calcein remaining in liposomes after partial adenovirus-mediated release could thus be calculated from the internal calcein concentrations predicted by the two hypotheses, using the standard calcein quenching curve determined previously (Allen, 1984). Table I shows examples of such experiments for different degrees of calcein release (columns 1 and 2). For each case, the level of calcein fluorescence expected by the all or none hypothesis equals the original (highly quenched) level multiplied by the fraction of vesicles that are intact (column 3). On the other hand, the level of calcein fluorescence expected for a graded release, in which the local calcein concentration inside the liposomes will decrease but the fluorescence per unit concentration of calcein will increase due to relief of self-quenching, is shown in column 4. To measure the level of calcein fluorescence for the marker remaining in the liposomes after adenovirus-induced leakage, the liposomes containing residual calcein were separated from released calcein on a PD-10 column, and their fluorescence was determined before and after addition of $C_{12}E_9$ (Table I, column 5). These results indicate that adenovirus-mediated release shows a better fit with the predicted values for the graded release hypothesis (column 4). This is in contrast to results with cytolysin (Blumenthal et al., 1984), which forms a large pore in the membrane and causes individual liposomes to lose their entire content.

Appearance of Liposomes Interacting with Adenovirus. To investigate further the nature of the lesion formed by the interaction of adenovirus and liposomes, we examined liposomes exposed to adenovirus by electron microscopy using negative staining. Figure 9 shows examples of SUV preincubated in the presence of glycerol at pH 5.6, followed by dilution in a glycerol-free medium (see Figure 8). The sample was concentrated by using a negative-pressure dialysis system. The typical icosahedron shape of the adenovirus particles was frequently found to have attached liposomes at the vertices (Figure 9A, arrowhead). In some cases, the liposomes were still intact and excluded stain (Figure 9B, arrowheads), but may appeared lysed and stained within (Figure 9B, arrowhead +). The fibers of adenovirus are very difficult to visualize with this negative stain, but occasional images showed faint impressions of fiber (Figure 9B, small arrows). The lysed vesicle in Figure 9B appears to be attached to the virus at the base of the fiber attachment, which is the same location as penton base protein. Other images show a repetitive liposome association with vertex regions, although the vertices are not always easily discernible (Figure 9C–H). In Figure 9E, two liposomes appear together at a small region near one vertex, suggesting that this site is favored for binding. The observation that some of the liposomes appear intact but most appear to be lysed as judged by the penetration of the negative stain is consistent with our data indicating that under a given set of conditions there is extensive binding of liposomes to the virus either with or without release of contents.

Discussion

Our results show that adenovirus mediated a pH-dependent permeability increase in liposomes. The pH dependence was similar to that for adenovirus entry into the cytosol from endocytic vesicles (Seth et at., 1984a). Our data are complicated by the fact that at low pH the virus self-aggregates. This property compelled us to carry out the experiments with

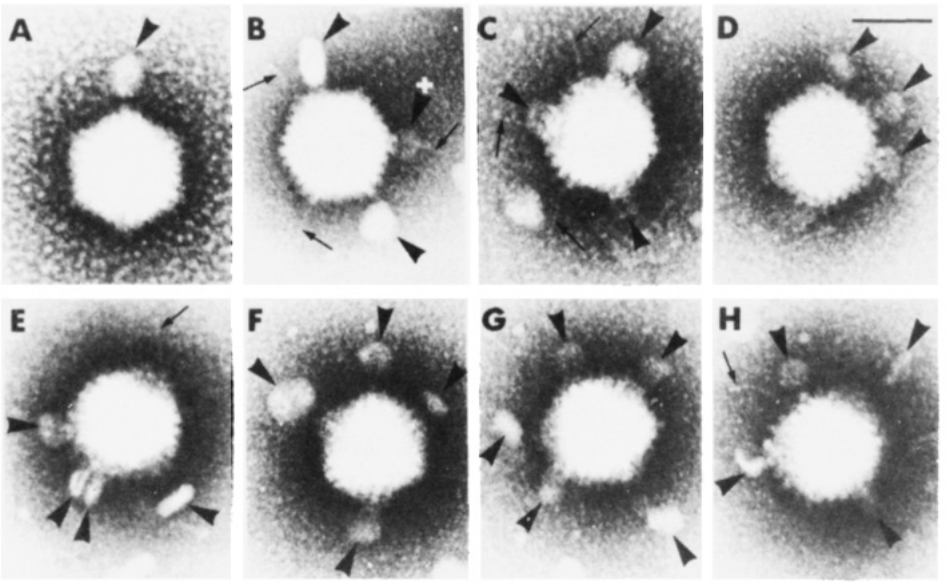

FIGURE 9: Appearance of liposome-treated adenovirus by negative-staining electron microscopy. Electron micrographs of samples of negatively stained egg PC SUV incubated with adenovirus in 30% glycerol–citrate, pH 6.1, and diluted into a glycerol-free buffer according to the protocol of Figure 8. The sample was concentrated in a negative-pressure dialysis apparatus and prepared for electron microscopy as described under Experimental Procedures. The typical icosahedron shape of the adenovirus particles was frequently found to have attached liposomes at the vertices (Figure 9A, arrowhead). In some cases, the liposomes were still intact and excluded stain (Figure 9B, arrowheads), but many appeared lysed and stained within (Figure 9B, arrowhead +). The fibers of adenovirus are very difficult to visualize with this negative stain, but occasional images showed faint impressions of fiber (Figure 9B, small arrows). The lysed vesicle in Figure 9B appears to be attached to the virus at the base of the fiber attachment, which is the same location as penton base protein. Other images show a repetitive liposome association with vertex regions, although the vertices are not always easily discernible (Figure 9C–H). In Figure 9E, two liposomes appear together at a small region near one vertex. Magnification 200000×; bar = 50 nm.

liposomes at the unphysiological temperature of 2 °C. Although adenovirus did induce release from liposomes at room temperature and neutral pH, the virus concentrations required for the neutral pH mediated release were an order of magnitude higher than those required for the low-temperature and low pH mediated release.

The experimental protocol of Seth et al. (1984c) to study adenovirus-induced permeabilization of plasma membranes was in part similar to the low-temperature protocol with liposomes. In the physiological experiments, self-aggregation was prevented by preincubating the virus for 60 min at 4 °C at pH 7.0 to secure binding to specific receptors. This was followed by washing and resuspending at the higher temperature and lower pH. In the liposome experiments, the membrane became sensitive to the disrupting action of adenovirus at the low temperature. However, when self-aggregation was prevented by preincubation with glycerol or sucrose, we observed a pH dependence at the temperature which reflects the physiological situation. Although it is well-known that liposomes are more easily destabilized than biological membranes, we feel that they are still reasonable models for endosome or plasma membrane.

Kjellen (1978) has shown that aggregated adenovirus is hemolytic and that the hemolysis is blocked by addition of an unspecified amount of liposomes. However, it is difficult to compare those data with our results for a number of reasons. (i) The amounts of virus used seemed to be in great excess over the amounts of target (about 10^6 virions per red blood cell). We observe maximal release at an adenovirus-lipid ratio of 10 which corresponds to about five liposomes per virion. (ii) The time it takes to release maximally is 2 h, whereas we obtain maximal release within 10 min. (iii) Hemolysis does not seem to be pH dependent.

By varying the structure of the lipids in the liposomes, we have concluded that lipid head-group structure plays at most a minor role in the ability of the virus to interact with membranes. On the other hand, our data show that when the fatty acid chains are fully saturated and in the solid phase, adenovirus mediates calcein release much less efficiently than when the chains are of a physiological composition.

When adenovirus interacts with a cell, it is initially bound to a specific receptor which binds to the fiber protein on the surface of the virus (Philipson et al., 1968). The virus–receptor complex is then internalized via coated pits into acidic endocytic vesicles termed receptosomes or endosomes. The virus rapidly escapes from the vesicles, apparently by causing their lysis (FitzGerald et al., 1983). The external proteins of adenovirus like many of the coat proteins of enveloped viruses (White et al., 1983) undergo a structural change when the pH falls to 5. All three capsid proteins of adenovirus develop an increased capacity to bind the nonionic detergent Triton X-114 at pH 5, suggesting that hydrophobic residues become exposed at the low pH (Seth et al., 1985). On the basis of these observations, we have suggested that adenovirus becomes inserted into the membrane of the endocytic vesicle at pH 5 and destabilizes the vesicle. The current studies which show that adenovirus can increase the permeability of liposomes at pH 5 are in accord with this hypothesis. We have noted that the penton base protein appears to play an especially important role in membrane lysis. This notion is based on the observation that antibodies to penton base block lysis of the endocytic vesicle but not adenovirus entry into the the vesicle (Seth et al., 1984b). Moreover, among the three major external proteins (hexon, penton base, and fiber), penton base acquires the greatest hydrophobic character at pH 5 as determined by its association with Triton X-114 (Seth et al., 1985).

The penton base proteins are found at the vertices of adenovirus, and the electron micrographs of adenovirus–liposome complexes indicate that lysed liposomes appear mainly attached to the vertices of the virus particles. It appears that the intact virus structure is required for membrane lysis. We found that release induced by empty adenovirus capsids was 5–10-

fold less efficient than release induced by intact virus. Moreover, neither intact penton base protein nor the other major external proteins (hexon, fiber) caused release of liposome contents (data not shown). Thus, it is unlikely that the penton base contains a pore-forming structure. This is consistent with our observation that release of marker from individual liposomes exhibited a graded rather than an all or none response, as was the case with pore-forming cytolysin (Blumenthal et al., 1984). Marker release is more likely to be caused by a transient perturbation of the lipid bilayer structure (Blumenthal & Klausner, 1982).

Since the fiber protein is bound to the receptor, the penton base is probably in close apposition to the target membrane. Lowering the pH in the endocytic vesicle could enable the hydrophobic residues in the penton base to react with a lipid patch as the initial event in target membrane disruption. The finding that adenovirus permeabilizes liposomes provides an assay which can now be used in an attempt to reconstitute the receptor into liposomes to study the entry process under conditions closer to those occurring in the cell.

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Myosin Subfragment 1 Has Tertiary Structural Domains†

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ABSTRACT: Transient electrical birefringence measurements were made on skeletal muscle myosin subfragment 1 (S1) at 3.7 °C in 10 mM tris(hydroxymethyl)aminomethane–acetate and 0.10 mM $MgCl_2$, pH 7.0. The specific birefringence for 4.5 μM S1 was determined from steady-state measurements to be $(8.1 \pm 0.3) \times 10^{-7}$ (cm/statvolt)^2. For electric fields in the range of 2.47–24.7 statvolts/cm, the alignment was due to a large permanent dipole moment for S1, estimated to be 8500 ± 2000 D. The duration and the strength of the transient electric field was varied, and the temporal response of the decay of the birefringence signal was analyzed. The rate of rotational motion after the field was removed increased with increasing field strength for short (0.35-μs) pulses and decreased with increasing pulse lengths for all field strengths. The rate of decay from a steady-state birefringence signal was independent of field strength. A model of S1 structure is proposed, which is consistent with these data and most other data on S1 structure. In this model, S1 is composed of two tertiary structural domains that are connected by a flexible linkage with a substantial restoring force. The electric dipole moments on the two domains are arranged head to tail. The segmental movement of the domains is restricted to certain directions. The average conformation of the molecule is elongated, but it can be made more compact by the torque exerted by an electric field. The structural changes depend on the strength and duration of the pulse. This hypothesized structure is discussed in regard to cross-bridge mechanisms of force generation that require S1 to bend and to an elastic element in the cross-bridge.

Force generation by actomyosin and ATP in muscle occurs while myosin is bound to actin. The portion of myosin that interacts with ATP and actin is called the cross-bridge, and a variety of cross-bridge mechanisms have been proposed for the force-generating step. These mechanisms vary widely in the details of the structural changes involved, including suggestions that the attached cross-bridge rolls, bends, or shrinks in order to cause the thick and thin filaments to interdigitate.

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