Membrane Fusion Correlates with Surface Charge in Exocytic Vesicles†

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Received December 22, 2003; Revised Manuscript Received April 16, 2004

ABSTRACT: Stimulation of gastric parietal cells results in exocytic recruitment of the proton pump (H^+,K^+-ATPase) from a pool of intracellular membranes (tubulovesicles) to the apical plasma membrane. We have previously reconstituted a step in this process, the homotypic fusion of tubulovesicles, and shown that they also fuse with liposomes in a protein-dependent manner [Duman, J. G., Singh, G., Lee, G. Y., Machen, T. E., and Forte, J. G. (2002) *Traffic 3*, 203−17]. Further, the lipid composition of the liposomes affects their ability to undergo fusion with tubulovesicles. In the present study, we investigated the lipid requirements for tubulovesicular membrane fusion using a fluorescent probe relaxation assay as well as transfer of protein between tubulovesicles and liposomes of defined composition. Initially, we tested the ability of tubulovesicles to undergo fusion with a panel of synthetic phosphatidylcholine-based liposomes containing a variety of common membrane lipids of various shapes and charges. We found that anionic lipids such as phosphatidylserine, phosphatidic acid, and phosphoinositides were best able to enhance tubulovesicle−liposome fusion and that they did it in a dose-dependent, apparently saturable manner. Next, we altered the lipid compositions of actual tubulovesicles and observed that addition of anionic lipids was able to enhance tubulovesicle−tubulovesicle fusion in vitro; thus, we hypothesized that the charge imparted by the lipids, per se, was responsible for the enhancement of membrane fusion. Accordingly, addition of negative charges to one of two pools of tubulovesicles in a fusion assay using anionic detergents increased membrane fusion; whereas, addition of positively charged cationic detergent decreased membrane fusion and could be used to back-titrate the anionic effects. Surprisingly, when both pools of fusing membranes were loaded with anionic detergents, fusion was markedly increased. The ability of anionic charges to enhance fusion was diminished as the ionic strength of the fusion medium was increased, suggesting that the mechanism of fusion enhancement depends on the surface charge of the membranes. Finally, the fusion reaction was highly dependent on temperature, and anionic charge appears to lower the activation energy of the fusion reaction. Taken together, these data suggest that (1) tubulovesicular fusion is enhanced by an increase in membrane surface negative charge associated with a lower activation energy and (2) neutralization or reversal of the surface charge prevents tubulovesicular fusion.

Eukaryotic cells are dynamic entities, constantly altering their form to carry out basic functions, both as unicellular organisms and within the context of multicellular organisms. In many cases, striking changes in cell morphology accompany changing functional activities of the cells. An excellent example of this phenomenon is the gastric parietal cell (*1*−*3*). Parietal cells are epithelial cells, located in the stomach, whose primary function is to secrete HCl into the gastric lumen in response to a meal. Stimulation of acid secretion is accomplished by the recruitment of proton pumps (H^+,K^+-ATPase) from a compartment of cytoplasmic membranes, called tubulovesicles, to the apical plasma membrane, resulting in a large expansion of surface area. In this configuration, the cell secretes large volumes of isotonic HCl, pH 0.8. This remarkable transformation is fairly rapid, happening on the order of minutes, and is fully reversible.

The regulation of membrane transporters by trafficking processes, first described in parietal cells (*2*), is a process shared with many other cell types (*4*, *5*). The mechanisms and components of the membrane fusion machinery identified in parietal cells are also related to those in other cells. The so-called SNARE proteins, VAMP-2 (*6*), SNAP-25 (*7*), and syntaxin 3 (*8*), are present in parietal cells and are involved in stimulatory exocytosis. Other proteins, such as Rab11a (*9*, *10*), IQGAP (*11*), syntaxin 1A (*12*), and SCAMPs (*13*), have also been implicated in this process as well. These findings underscore the utility of the parietal cell as a model for the more generalized process of exocytosis.

While identification and characterization of the proteic fusion machinery is forthcoming, much less attention has been given to the role(s) played by lipids in exocytosis. As the major structural component of membranes, lipids are obviously important in membrane fusion processes, but it remains unclear the extent to which lipids participate. Do lipids contribute properties, other than structural, to the membranes that influence their dynamics, or are they inert building blocks that are pulled along by an active protein machinery? As is the case with many membranes, H^+,K^+-ATPase-rich tubulovesicles in parietal cells are dominated

† Supported by NIH Grant DK10141.

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10.1021/bi036304q CCC: $27.50
Published on Web 05/22/2004

by phosphatidylcholine (PC)[1] and phosphatidylethanolamine (PE), with somewhat lesser quantities of phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (*14*, *15*). The small amounts of phosphatidic acid (PA) are obvious by virtue of its high turnover (*15*). PC and sphingomyelin reside chiefly in the extracytosolic membrane leaflet, while PS and PE reside in the cytosolic leaflet (*14*, *16*, *17*). In a previous study, we reconstituted the fusion of gastric tubulovesicles in vitro, using an R18-based dequenching assay and observed that it can be triggered by either Mg^{2+}/ATP or Ca^{2+} (*18*). In the case of Mg^{2+}/ATP-mediated fusion, the process specifically required Mg^{2+}/ATP, as Mg^{2+} alone or other Mg^{2+}/nucleotide complexes were unable to support fusion; also, chelation of Mg prevented fusion, even in the presence of ATP. Moreover, ATP hydrolysis was required, as a nonhydrolyzable ATP analogue was unable to support fusion. Fusion did not require functional H^+,K^+-ATPase, despite the fact that it is the predominant ATPase in these membranes. In that study, we also demonstrated that tubulovesicles can be triggered to fuse with protein-free liposomes (*18*). Strikingly, the ability of tubulovesicle membranes to undergo fusion with liposomes depends on the composition of the liposomes. Liposomes comprised of the diverse lipids extracted from tubulovesicles make excellent fusion substrates, while liposomes comprised of pure dioleylphosphatidylcholine (DOPC) do not (*18*). This suggests that the lipid components of the fusing membranes radically affect fusion.

In this study, we examined the fusion of tubulovesicles with a variety of synthetic liposomes. We then manipulated the lipid composition of native membranes, and determined that, in fusion of tubulovesicles with liposomes as well as with other tubulovesicles, anionic phospholipids favor membrane fusion. We then addressed the mechanism of this phenomenon by loading membranes with ionic detergents and determined that negative surface charge, per se, is critical for membrane fusion. Finally, we performed a number of experiments to determine the nature of this requirement and verify these results using alternate assays. Our results indicate that negative surface charge is required for a rate-determining step in membrane fusion and that anionic phospholipids may also serve some other role, probably that of providing binding sites for protein components.

MATERIALS AND METHODS

Isolation and Preparation of Gastric H^+,K^+-ATPase-Rich Tubulovesicles. Tubulovesicles were isolated from homogenates of New Zealand White rabbit stomachs using differential centrifugation and density gradient flotation as previously described (*19*). There was no observable difference between the behavior of the membranes from the 38/30% interface and the 30/10% interface, so the data have been reported for both, collectively termed tubulovesicles. After isolation, the tubulovesicles were stored at 1 mg/mL protein. Since membrane lipid (phospholipid + cholesterol) is equivalent to membrane protein in tubulovesicles (*20*), this is approximately 2 mg/mL total membrane weight. Throughout the paper we refer to the concentration of membranes in terms of mg protein/mL. Prior to use, isolated tubulovesicles were routinely tested for their ability to transport protons in a K^+-dependent manner (*21*) and were also probed by Western blot for the presence of H^+,K^+-ATPase using the monoclonal antibody 2G11 (*22*). All procedures involving the procurement of animal tissues were reviewed by the Berkeley Animal Care and Use Committee.

Labeling of Tubulovesicles with R18 and R18 Fusion Assays. Octadecylrhodamine chloride (R18) was introduced into tubulovesicles following the procedure previously developed (*18*). The principle of the R18 fusion assay is that membranes are loaded with concentrations of the dye that lead to self-quenching. When these donor membranes fuse with unlabeled acceptor membranes, the dye is diluted, self-quenching decreases, and an increased R18 emission is observed. All fusion/dequenching assays were performed in a Spex fluorimeter exciting at 560 nm and monitoring at 590 nm. We generally performed the assays at 37 °C in fusion buffer (FB: 10% sucrose, 5 mM HEPES, pH 7.0) in 5 mm optical glass cuvettes. In experiments containing NaCl or KCl, the fusion buffer consisted of the indicated [salt] in the same buffer, with enough sucrose present so that the salt solutions were isotonic to FB. Total reaction volume was 350 μL. We typically added membranes containing 1 μg of protein of R18-labeled donor membranes and 19 μg of protein unlabeled acceptor membranes. After a short time of relatively stable fluorescence (e.g., 30 s), fusion was triggered by adding either 2 mM Mg^{2+}/ATP or 1 μM Ca^{2+}. Ca^{2+} was buffered to 1 μM using Ca^{2+}/CaEGTA (*18*). At the end of each assay (2.5 min after addition of the trigger), fusion mixtures were solubilized with 0.2% TX-100 to determine the total fluorescence. Total R18 fluorescence of each sample was used to adjust relative fusion rates for individual treatments and as a standard to determine total rounds of fusion/s (see Results)

Preparation of Liposomes. All phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL) or Sigma-Aldrich (St. Louis, MO), including dioleylphosphatidylcholine (DOPC), 1,2-dioleyl-*sn*-glycero-3-ethylphosphocholine ($DOPC^+$), dioleylphosphatidylethanolamine (DOPE), dioleyl−diacylglycerol (DODAG), dioleylphosphatidyl serine (DOPS), dioleylphosphatidic acid (DOPA), dipalmitoylphosphatidic acid (DPPA), phosphatidylserine with mixed fatty acid chains isolated from bovine brain (brain PS), phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI_4P), phosphatidylinositol-4,5-(bis)phosphate ($PI_{4,5}P_2$), lysophosphatidylethanolamine (lysoPE), and lysophosphatidylcholine (lysoPC). Synthetic liposomes were produced by mixing chloroform solutions of the indicated phospholipids together in Pyrex tubes and then gently evaporating the solvent under N_2. The resulting lipid films were placed under vacuum for 10 min to completely remove all chloroform. The lipid films were then suspended in FB to a concentration of 2 mg/mL and sonicated on ice with a probe tip sonicator, using five pulses of 10 s each. A total of 38 μg of liposomes served as acceptors in fusion assays.

[1] Abbreviations: R18, octadecylrhodamine chloride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PI_4P, phosphatidylinositol 4-phosphate; $PI_{4,5}P_2$, phosphatidylinositol-4,5-(bis)phosphate; DOPC, dioleylphosphatidylcholine; $DOPC^+$, 1,2-dioleyl-*sn*-glycero-3-ethylphosphocholine; DOPE, dioleylphosphatidylethanolamine; DODAG, dioleyl-diacylglycerol; DOPS, dioleylphosphatidyl serine; DOPA, dioleylphosphatidic acid; DPPA, dipalmitoylphosphatidic acid; lysoPE, lysophosphatidylethanolamine; lysoPC, lysophosphatidylcholine; OSGP, octyl-β-D-thioglucopyranoside; RhDHPE, Lissamine rhodamine *B*-1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine.

H^+,K^+-ATPase Transfer Assay for Fusion. Alternate assays to measure fusion by the transfer of protein from tubulovesicular donors to liposomal acceptors were performed as previously described (*18*). Twice the usual amount of tubulovesicular donors and liposomal acceptors were combined (acceptors were omitted in some assays as noted), and the standard fusion reactions were carried out as stated previously. Following completion of the assay, the membrane mixtures (in normal 10% sucrose fusion buffer as stated previously) were layered onto 20% sucrose cushions. These minidensity gradients were spun for 90 min at 4 °C at 170 000*g* in a Beckman TLS55 rotor. Fractions were collected from the gradients (the upper 10% sucrose layer; the interface between 10 and 20% sucrose; the 20% sucrose layer; and the pellet) and loaded onto nitrocellulose membranes using a slot-blot apparatus (Hoefer Scientific Instruments, San Francisco, CA) and washed with deionized water. The membrane was blocked with 5% milk in PBS and probed with an anti-H^+,K^+-ATPase antibody (2G11, ABR, Boulder, CO). The probe was detected using enhanced chemiluminescence (Renaissance, NEN Life Science Products, Boston, MA).

Addition of Exogenous Lipids into Tubulovesicles by Chloroform Incubation. Exogenous lipids were added to tubulovesicles by adding lipid stocks in chloroform to membranes while vortexing. The amount of chloroform in the sample never exceeded 3−5% of the total volume, and controls with solvent alone were run along with all lipid additions. After ∼20 s of vortexing, the membranes were gently shaken at 4 °C for 30 min. Membranes were then washed in suspending medium (SM: 10% sucrose, 0.4 mM EDTA, 5 mM Tris, pH 7.4) and pelleted by centrifugation at 231000*g* for 25 min. Membranes were gently resuspended to 1 mg/mL in SM.

Detergent Solubilization and Reconstitution of Membranes. If exogenous lipids were to be added, these were first dried into a film in an Eppendorf tube using N_2 and vacuum as stated previously; otherwise, this step was omitted. Tubulovesicles were then added in SM at a concentration of 1 mg/mL. The 13.5 mM octyl-β-D-thioglucopyranoside (OSGP, CalBiochem, San Diego, CA) was then added, and the sample was vortexed for 15 s and then shaken gently at 4 °C for 1 h. (The critical micelle concentration of OSGP is 9 mM.) Samples were then diluted 4-fold with SM while vigorously vortexing. The membranes were then washed, pelleted, and resuspended in the same manner as the chloroform incubated membranes.

Determination of Phospholipid Loading Efficiency by the Previous Methods. A mixture of 90% DOPC and 10% Lissamine rhodamine B-1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (RhDHPE, Molecular Probes, Eugene, OR) was added to tubulovesicles either by incubation with chloroform or by detergent reconstitution (see previous). For the chloroform method, the amount of DOPC/RhDHPE was equal to either 10 or 20% of the total lipid (DOPC/RhDHPE + tubulovesicular lipid) in the sample; for the OSGP method, it was equal to 10% of the total lipid. After introduction of the lipid, membranes were washed. The pellet and supernatant were separated, and the pellet was resuspended to equal the volume of the supernatant. Both pellet and supernatant were solubilized with 0.3% TX-100, and the RhDHPE fluorescence was read in a Spex fluorimenter (E_x = 561 nm, E_m = 582 nm). Results were expressed in the percentage of RhDHPE fluorescence recovered in each fraction out of total fluorescence in a single loading experiment. Protein recovery was also measured using the Bradford assay.

Loading of Ionic Detergents into Tubulovesicles. Ionic detergents were added from 10× stock solutions. They were added to tubulovesicles while vortexing to minimize exposure of membranes to detergent concentrations above the critical micelle concentration. In membranes that were loaded with more than one detergent, the detergents were added sequentially in this first step. After addition of detergents, the membrane/detergent mixtures were incubated at 37 °C for 20 min. At this point, loaded membranes were used as acceptors in membrane fusion assays or labeled with R18 for use as donors. We sometimes pelleted and washed the membranes as stated previously after detergent loading, but we found that this treatment did not change the results of the fusion assays, so it was not routinely done.

Static Light Scattering. Light scattering measurements were used to assess the solubilization of membranes by detergents. A total of 30 μg of tubulovesicles were suspended in 270 μL of FB in an optical glass cuvette at 37 °C. Light scattering was monitored by exciting at 590 nm and monitoring at 600 nm using a Spex fluorimenter. SDS was added at various concentrations and the degree of scattering measured for ∼1 min. Clarification of the solution, manifested by a decrease in light scattering, indicates dissolution of membranes.

Measurement of E_a. The activation energy (E_a) was obtained by performing fusion assays at various temperatures using untreated tubulovesicles mixed with normal untreated acceptors or acceptors loaded with 10 or 100 μM SDS. The initial rate (V_i) of R18 dequenching in ΔE_{m590}/s was obtained by applying a linear fit to the first 12 s of accessible points post-triggering with 2 mM Mg^{2+}/ATP. These linear fits were excellent, except at very low temperatures where the very low rates created some noise. Because of the large excess of acceptors in each fusion assay, we were able to assume that R18-containing membranes fuse predominantly with unlabeled membranes through many rounds of fusion. R18 fluorescence was determined before and after TX-100 solubilization to determine the relationship between dilution of a given amount of R18 in the membranes and its apparent fluorescence as a percentage of the total fluorescence in the sample (relative quantum yield). Relative quantum yield depended linearly on R18 dilution in the membrane, as previously reported (*18*), and the linear relationship obtained from SDS-loaded membranes did not differ significantly from data obtained from untreated membranes (data not shown). The total amount of R18 fluorescence in each fusion assay was used to convert V_i from ΔE_{m590}/s to relative quantum yield/s. V_i was taken to be the first-order rate constant for this process (k). E_a was determined by plotting ln k as a function of T^{-1} (in degrees Kelvin), which yields a line described by the Arrhenius equation

$$\ln k = -\left[\frac{E_a}{R}\frac{1}{T}\right] + \ln A \qquad (1)$$

where R is the gas constant and A is the preexponential factor, which correlates with the change in entropy for the reaction.

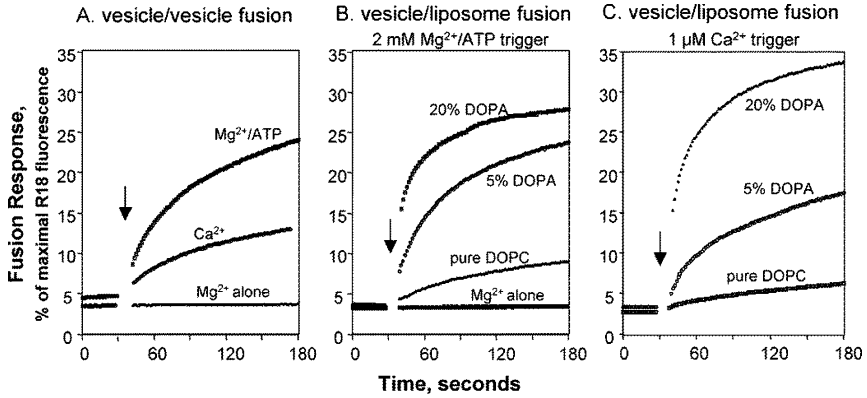

FIGURE 1: Tubulovesicles fuse with other tubulovesicles and with liposomes in a Ca^{2+}- or Mg^{2+}/ATP-dependent manner. Tubulovesicle donors were loaded with R18 as described in Materials and Methods, and at zero time, mixed with acceptors consisting of either other tubulovesicles (A) or liposomes (B and C). (A) Tubulovesicle−tubulovesicle fusion. The arrow indicates addition of 2 mM Mg^{2+}/ATP, 1 μM Ca^{2+}, or Mg^{2+} alone, as indicated. The fluorescence falls to zero during the time the shutter is open. (B) Tubulovesicle−liposome fusion triggered by Mg^{2+}/ATP. At zero time, tubulovesicle donors were mixed with DOPC liposomes containing 0, 5, or 20% DOPA as indicated. After equilibration for about 30 s, 2 mM Mg^{2+}/ATP was added to trigger fusion (arrow). Tests were also run to determine if fusion could be triggered by 2 mM $MgSO_4$ without ATP (Mg^{2+} alone). In these cases, fluorescence did not change and the lines for 0, 5, or 20% DOPA were indistinguishable at this resolution. (C) Tubulovesicle−liposome fusion triggered by 1 μM Ca^{2+}. Tubulovesicle donors were mixed with DOPC liposomes containing 0, 5, or 20% DOPA, and fusion was triggered by 1 μM Ca^{2+} (arrow).

The slope of the line, therefore, is equal to $-E_a/R$. The experiments were repeated with good reproducibility.

EM-Based Assay for Fusion. Two populations of tubulovesicles were labeled: one was incubated in culture supernatant containing a monoclonal antibody (2G11) against the β-subunit of H^+,K^+-ATPase, then washed with SM; the other was loaded with 10% of the phospholipid PE-Biotin Cap (Avanti Polar Lipids, Alabaster, AL) by the chloroform method and then washed. Both membranes were resuspended in SM to 1 mg protein/mL. We coincubated equal amounts of each membrane at 37 °C for 5 min in the absence or presence of a fusion trigger; the trigger was either 2 mM Mg^{2+}/ATP or 1 μM Ca^{2+}. Membranes were then pipetted onto Formvar-coated nickel grids, and the liquid was carefully blotted off 40 s later. The grids were dried, then blocked for 15 min in 5% BSA in PBS, and stained with a combination of 15 nm gold-conjugated goat-antimouse IgG and 10 nm gold-conjugated streptavidin (nominal sizes, both from Ted Pella, Inc., Redding, CA). The grids were then washed 7 times with blocking solution. Membranes were negative stained using 2% phosphotungstic acid, pH 7.0, and visualized with a Tecnai 12 electron microscope (FEI, Eindhoven, The Netherlands) operating at 100 kV. Micrographs of membranes were then scored as to whether they had no labels associated, one or the other label associated, or both labels associated. The two gold particles, 10 and 15 nm, were easily distinguished from each other. A total of 2800 membranes were scored by two separate investigators. For a membrane to be scored as labeled, the gold particle had to be within one membrane thickness of the membrane. For some labels, it was unclear as to which membrane it was associated. These labels were excluded from scoring. Membrane surfaces could only be scored if the membrane itself was clearly visible and sufficiently distinct from other membranes.

Data Analysis. Data from the fusion assay are generally presented as relative fusion response (sometimes called fusion response). This number is the difference between the R18 fluorescence 2.5 min after triggering and the average R18 fluorescence prior to triggering. Control experiments consisting of untreated donors and acceptors triggered with 2 mM Mg^{2+}/ATP were assigned a value of 1, and the remaining experiments were normalized accordingly. Where indicated, results are reported as the mean ± SEM. Student's *t*-test was used to determine statistical significance, using paired data for the analysis when available. P values <0.05 were considered significant.

RESULTS

Fusion of Tubulovesicles and Liposomes. Previous work showed that tubulovesicle membranes fuse with liposomes in both a Ca^{2+}-dependent and a Mg^{2+}/ATP-dependent manner (*18*). In that study, liposomes made from lipids extracted from tubulovesicular membranes fused well with tubulovesicles; whereas, liposomes made from DOPC were poorly fusogenic. Examples of the fusion reaction between tubulovesicle membranes as donors and tubulovesicles or liposomes as acceptors are shown in Figure 1. In all cases, the fluorescence baseline was stable until the addition of a fusion trigger (indicated by arrows in each panel). Panel A of Figure 1 shows the reaction between tubulovesicular donors and tubulovesicular acceptors. In agreement with our previous results, Ca^{2+} and Mg^{2+}/ATP triggered robust increases in R18 fluorescence that correlate with membrane fusion (*18*). The Mg^{2+}/ATP-triggered response was more pronounced than the Ca^{2+}-triggered response and required ATP, as Mg^{2+} alone was not sufficient. Panel B of Figure 1 shows the Mg^{2+}/ATP-triggered fusion reactions between tubulovesicular donors and liposomal acceptors made of DOPC. Once again, Mg^{2+} alone was insufficient to produce the fusion response observed in the presence of Mg^{2+}/ATP. Addition of differing concentrations of PA into the DOPC-containing liposomes yielded a dose-dependent increase in the observed fusion response (see next). Panel C of Figure 1 is similar to panel B except that Ca^{2+} replaced Mg^{2+}/ATP as the fusion trigger. The fusion response increased in a dose-dependent manner as PA was added to the liposomes. These data reflect the type of raw data that we collected throughout this study and are reported either as the fusion response or the initial rate of fusion (see Materials and Methods).

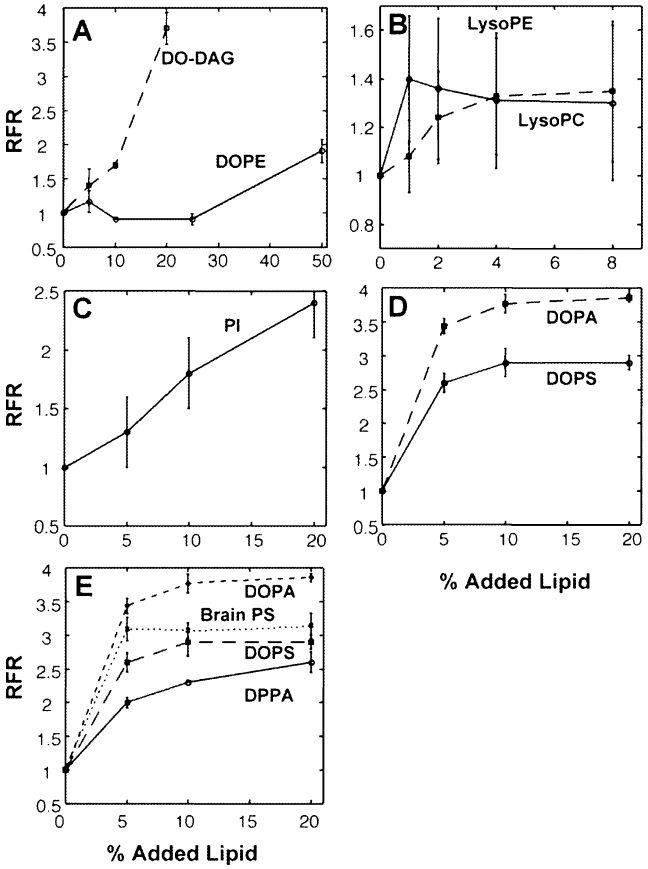

FIGURE 2: Effect of liposome composition on the fusion reaction between tubulovesicles and liposomes. Liposomes consisting of dioleylphosphatidylcholine (DOPC) and the indicated amounts of various synthetic phospholipids were used as acceptors in fusion experiments with tubulovesicle donors. The fusion response was calculated 2.5 min after triggering with 2 mM Mg^{2+}/ATP. A value of 1 represents the fusion of tubulovesicles with liposomes consisting of 100% DOPC (usually less than 5% of total fluorescence). Panel A shows the addition of neutral cone-shaped lipids, dioleyl−diacylglycerol (DO−DAG), and dioleylphosphatidylethanolamine (DOPE). Panel B shows the addition of neutral inverted cone-shaped lysolipids, lysophosphatidylethanolamine (lysoPE), and lysophosphatidylcholine (lysoPC). Panel C shows the addition of phosphatidylinositol (PI). Panel D shows the addition of more strongly anionic phospholipids, dioleylphosphatidic acid (DOPA), and dioleylphosphatidyl serine (DOPS). Panel E compares the effects of anionic phospholipids having differing fatty acid chains, and panel F shows the addition of a cationic phospholipid, dioleylphosphatidyl ($DOPC^+$). All results shown ±SEM ($n \geq 3$).

We proceeded to test the effects of lipids on the fusion reaction by examining fusion between tubulovesicles and synthetic liposomes of varying composition. Our strategy was to introduce other lipids into synthetic DOPC-based liposomes and assay their ability to increase fusion with tubulovesicles in response to Mg^{2+}/ATP. Our readout was the R18 assay, with tubulovesicles serving as R18 donors and liposomes serving as acceptors. In selecting the lipids, we considered both the shape and the charge of the lipids added to the DOPC liposomes. DOPC itself is a neutral, cylindrical lipid. Initially, we added neutral lipids with small headgroups (cone-shaped) such as DOPE and DODAG. We observed an increase in the fusion activity with tubulovesicles that was dependent on the dose of cone-shaped lipid (Figure 2A). DODAG, which has the smaller of the two headgroups, was markedly more effective at increasing fusion at concentrations greater than 10% of total lipid. We then examined the effect of neutral lipids with only one fatty acid side chain, giving them an inverted cone shape, LysoPE and LysoPC. In amounts up to 8% of total lipids, these lysolipids were not effective at altering fusion (Figure 2B). We were unable to add lysolipids to liposomes in as great proportions as the other lipids due to their detergent-like behavior, which interfered with the R18 signal.

We next tested the effects of negatively charged phospholipids. PI, which has a neutral headgroup despite its overall negative charge, significantly increased fusion in a dose-dependent manner (Figure 2C). DOPA and DOPS, anionic lipids with charged headgroups, also increased fusion, but the dose dependence was markedly different from that of PI. Not only did these lipids increase the fusion to a higher extent and at lower relative concentrations than PI, but their effect seemed to saturate at around 10% of the total liposomal lipid (Figure 2D). DOPA enhanced fusion significantly better than DOPS. Up to this point, we had been primarily adding lipids with oleic acid side chains. To investigate the effect of changing the interior (fatty acid side chains) of the membrane as well as the surface (headgroups), we compared the fusion activities imparted by DOPA and DOPS with those imparted by DPPA and by PS extracted from brain, which has a mixture of side chains. Overall, the shapes of the dose−response curves of all four composite liposomes were quite similar, although DPPA was slightly less effective at promoting fusion than DOPA (Figure 2E). These results indicate that while the ability of a lipid to affect tubulovesicle−liposome fusion may be influenced by hydrocarbon tails, the surface of the membrane is actually a more important determinant of its fusibility. Results obtained with Ca^{2+}-triggered fusion were similar in all cases (data not shown).

Assessment of Fusion by Incorporation of Tubulovesicle Protein into Liposomes. All previous experiments measuring fusion between tubulovesicles and liposomes of varying composition were performed using the R18 as a fusion reporter in the system. To address the question of a possible R18 artifact, we performed alternate assays that allow us to monitor fusion by protein transfer without using R18 (*18*). Because liposomes are much less dense than tubulovesicles, they fail to penetrate a 20% sucrose cushion, while most tubulovesicles do. When tubulovesicles fuse with an excess of liposomes, most of the resulting fusion products are unable to penetrate the cushion as well. We can detect these fusion products by assaying the fractions of a simple step gradient for presence of H^+,K^+-ATPase, which is resident in tubulovesicles and whose 11 transmembrane domains prevent it from being removed from the membranes. This assay allowed us to show that our fusion reactions occur independently of the presence of R18 in the membranes at all and allowed us to rule out collisional transfer of R18 as an explanation of the results.

In the present experiments, we mixed tubulovesicles in assay concentrations at 37 °C with either pure DOPC liposomes or liposomes containing 10% DOPA and 90% DOPC. As an additional control, tubulovesicles alone were incubated without any acceptor liposomes. Three minutes after adding Mg^{2+}/ATP to trigger fusion, the mixtures were separately layered onto 20% sucrose cushions and centrifuged. Fractions were collected, applied to nitrocellulose using a slot-blot apparatus, and immunoassayed for H^+,K^+-

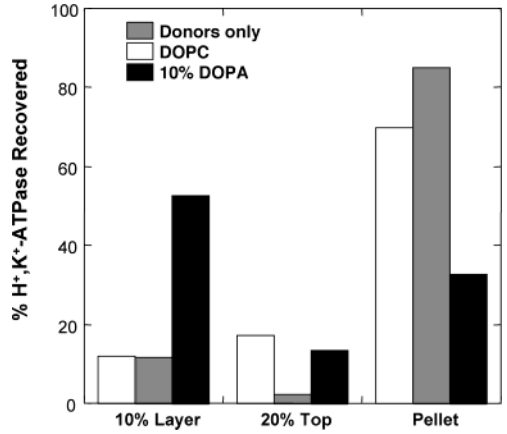

FIGURE 3: Fusion assay in the absence of R18 confirms the anionic effect. Tubulovesicles that were not loaded with R18 were used as donors in fusion assays with liposomal acceptors. Fusion reactions, triggered by 2 mM Mg^{2+}/ATP, were carried out between these membranes mixed with 100% DOPC liposomes (DOPC), DOPC liposomes containing 10% DOPA (10% DOPA), or no acceptors as a control (donors only) as described in Materials and Methods. At the end of the incubation, the resulting fusion mixtures in 10% sucrose fusion buffer were layered onto 20% sucrose cushions and spun at 170 000*g* for 2.5 h. Fractions were collected from the gradient as follows: 10% layer, including all material in the 10% layer and at the 10−20% interface; 20% top, including all material suspended in the 20% layer; and pellet, material that sedimented as a pellet. Fractions were loaded onto nitrocellulose with a Slot-Blot apparatus and probed with the monoclonal anti-H^+,K^+-ATPase 2G11. After development using enhanced chemiluminescence, R18 signal was quantified by densitometry using NIH Image. The results shown here are representative of five independent experiments.

ATPase. The results in Figure 3 show that when tubulovesicular donors were incubated without acceptor liposomes, the large majority of H^+,K^+-ATPase (~80%) penetrated the cushion and was recovered in the pellet. In contrast, the DOPA-containing membranes caused a Mg^{2+}/ATP-dependent shift of H^+,K^+-ATPase to the lighter fractions. About half of the H^+,K^+-ATPase did not penetrate the cushion and was recovered in the 10% layer, consistent with earlier results using liposomes made from tubulovesicular lipids (*18*). In fusion conditions using liposomes made with 100% DOPC, the distribution of H^+,K^+-ATPase was much more oriented to the heavy fractions, with only about 10% remaining in the 10% layer, indicating that fusion occurred to a much lesser extent than with added DOPA. These results support the R18 data concerning enhanced fusion with anionic liposomes.

Fusion of Tubulovesicles with Tubulovesicles Having Exogenously Added Lipids. To determine whether the effects of various phospholipids on tubulovesicular−liposomal fusion were relevant to fusion in biological membranes, we tested the ability of unmodified tubulovesicles to fuse with tubulovesicles having altered phospholipid compositions. In one approach, we introduced various lipids to tubulovesicles by incubating the membranes with chloroform solutions of lipids, then washing them. Two different amounts of lipid were loaded into the membranes: one corresponding to 10% of the total lipid in the sample and one corresponding to 20% of the total lipid. We then used these modified membranes as acceptors against untreated donors in Mg^{2+}/ATP-dependent fusion assays. The results of these experiments are shown in Figure 4A. Incubation of tubulovesicles with chloroform alone produced no significant effect on

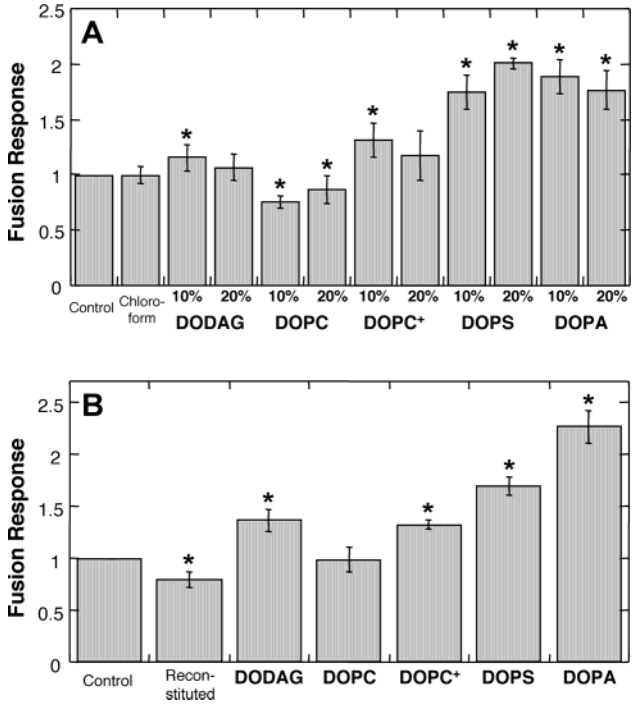

FIGURE 4: Modifying the lipid content of tubulovesicles alters tubulovesicle−tubulovesicle fusion response. In panel A, the indicated phospholipds were introduced into tubulovesicles using the chloroform incubation method (see Materials and Methods). Lipids were added in two different amounts, corresponding to 10 and 20% of the total lipid in the modified tubulovesicles. In fusion assays, the modified tubulovesicles served as acceptors to normal tubulovesicle donors. Fusion response was measured 2.5 min after triggering with 2 mM Mg^{2+}/ATP. The control bar indicates the response when unmodified tubulovesicle acceptors were used, and the chloroform bar represents a solvent control in which the tubulovesicle acceptor membranes were loaded with chloroform containing no phospholipids. In panel B, the membranes were loaded with amounts of phospholipid corresponding to 10% of the total lipid using the reconstitution method (see Materials and Methods), but otherwise the experiments are the same. The reconstituted bar represents an experiment in which the tubulovesicle acceptors were reconstituted, but no exogenous phospholipids were added in the process. All data are shown ±SEM ($n \geq 3$). An asterisk indicates significant difference from control ($P < 0.05$).

fusion with untreated tubulovesicular donors. Addition of DOPC mildly suppressed fusion relative to the control, and the positively charged lipid $DOPC^+$ mildly increased fusion. DODAG also had a mild stimulatory effect. The addition of negatively charged lipid caused marked increases in the fusion responses. Both DOPA and DOPS almost doubled the fusion response. In none of these cases did any of the lipids show a dose dependence in our assays, indicating that, if there was any dose dependence, our membranes were saturated at the amounts of added lipid. Further, in no case was any trigger-independent fusion observed.

To confirm these results, exogenous lipids were introduced into tubulovesicles using an alternative method. Tubulovesicles were solubilized in the presence (or not) of lipid films using the detergent octyl-β-D-thioglucopyranoside (OSGP). The amount of lipid in the film corresponded to 10% of the total lipid in the sample. Membranes were reconstituted by suddenly diluting the samples, dropping [OSGP] beneath its critical micelle concentration, which caused the membranes to reform. The membranes were then harvested by centrifugation, washed, and used as acceptors

Table 1: Addition of PI and PI Phosphates to Tubulovesicles[a]

condition	fusion response (SEM)
control	1.00 ± 0.00
chloroform blank	1.01 ± 0.08
PI (10%)	1.12 ± 0.09^b
(20%)	1.70 ± 0.14^b
PI_4P (10%)	1.74 ± 0.12^b
$PI_{4,5}P_2$ (10%)	1.60 ± 0.12^b
(20%)	1.19 ± 0.27

[a] Tubulovesicles were loaded with the indicated PI or the indicated PI phosphate using the chloroform addition method described in Materials and Methods. PI and PI phosphates were added to the indicated percentage of total membrane lipid. Loaded tubulovesicles were used as acceptors with untreated tubulovesicular donors in fusion reactions that were triggered with 2 mM Mg^{2+}/ATP. Fusion response was set to 1.00 for the control (untreated tubulovesicles used as acceptors). Results are shown ±SEM ($n \geq 3$). [b] Significant difference between the sample and the chloroform blank ($P < 0.05$).

against normal tubulovesicular donors. The results of these experiments are shown in Figure 4B. Reconstituted membranes without any exogenously added lipid exhibited somewhat lower fusibility than untreated membranes. Reconstitution in the presence of DOPC raised fusibility slightly but not significantly. Adding DODAG or $DOPC^+$ caused a small increase in fusion. Once again, the addition of negatively charged lipids had the most pronounced effects. DOPA was the most potent fusion enhancer, although addition of DOPS did increase the fusion activity over the samples reconstituted without any exogenous lipid to >150%.

Phosphoinositides were also added to the membranes using the chloroform addition method (Table 1). In contrast to DOPS and DOPA, and consistent with Figure 2C, addition of PI affected the fusion response in a dose-dependent manner over the range we used. A concentration of 20% PI had an especially marked effect on fusion, increasing it by ~175%, while 10% PI had only a small effect. PI_4P, which consists of PI with the 4- I loaded atposition on the inositol ring phosphorylated, was as effective at 10% as P 20%. $PI_{4,5}P_2$, which is like PI_4P but with the 5-position also phosphorylated, was no more effective than PI_4P in increasing fusion response, and higher amounts (e.g., 20%) of this lipid actually caused the fusion response to decrease. These results, taken together with those in Figure 4, indicate that a negative charge in the membrane facilitates membrane fusion.

An issue of concern regarding the addition of lipids is the efficiency with which they are incorporated. To address this question, we loaded tubulovesicles with a mixture of DOPC and a rhodamine B-labeled derivative of DHPE (RhDHPE) as a tracer probe. When tubulovesicles were loaded, either by the chloroform incubation method or by OSGP reconstitution, this tagged lipid allowed us to trace its association with the membranes. As shown in Table 2, the chloroform method was fairly efficient in its ability to introduce lipids to the membranes. Approximately 80% of the added label became associated with the membranes regardless of the total amount of lipid used. This necessitates a slight correction in expressing the amount of lipid added as total lipid: instead of 10 and 20%, 8 and 16% may be closer to the actual values. By contrast, OSGP reconstitution was much less efficient, as only about one-third of the added lipid became associated with the membranes. Using the Bradford assay as a readout

Table 2: Introduction of Lipids into Tubulovesicles by Different Methods[a]

condition	amount of lipid (%)	% label in pellet	% label in supernatant
chloroform	10	77.2 ± 7.6	22.9 ± 7.7
	20	81.0 ± 9.4	19.0 ± 9.4
OSGP	10	33.0 ± 1.4	66.9 ± 1.4

[a] A mixture of 90% DOPC and 10% RhDHPE was added to tubulovesicles either by incubation with chloroform or by reconstitution with OSGP (see Materials and Methods). For the chloroform method, the amount of DOPC/RhDHPE was equal to either 10 or 20% of the total lipid (DOPC/RhDHPE + tubulovesicular lipid) in the sample; for the OSGP method, it was equal to 10% of the total lipid. After introduction of the lipid, membranes were washed. The pellet and supernatant were separated, and the pellet resuspended to equal the volume of the supernatant. Both were solubilized with 0.3% TX-100, and the RhDHPE fluorescence was read ($E_x = 561$ nm, $E_m = 582$ nm). Results are expressed as the percentage of RhDHPE fluorescence recovered in each fraction out of total fluorescence in a sample. Results are shown ±SEM ($n = 3$).

of our protein recovery, we determined that protein recovery in the chloroform-loading condition was almost complete and that protein recovery in the OSGP reconstitution experiments was generally close to 75%. As significant effects were seen under these reconstitution conditions, the results of RhDHPE tracking further support the idea that the amounts of lipids added by the chloroform method were saturating with regard to fusion activity.

EM-Based Assay of Membrane Fusion. Because of the variability in tubulovesicle size and several other technical problems (*23*), it has been difficult to assess vesicular fusion by electron microscopy. However, the ability to introduce membrane tags allowed us to develop an EM based assay to assess the validity of tubulovesicular fusion. Our strategy was to introduce different labels into populations of donor and acceptor membranes in the total absence of R18 and determine whether the labels colocalized on fused membranes. One population of tubulovesicles was labeled with an antibody against the cytosolic epitope of H^+,K^+-ATPase, and another population was loaded with biotinylated PE, as described in the Materials and Methods. After mixing the two populations at 37 °C in the presence of trigger (either 2 mM Mg^{2+}/ATP or 1 μM Ca^{2+}) or no trigger, the membranes were laid onto nickel grids and stained with goat anti-mouse IgG conjugated to 15 nm gold to detect H^+,K^+-ATPase and streptavidin conjugated to 10 nm gold to detect biotinylated PE. The membranes and labels were then visualized by negative staining with phosphotungstic acid.

Almost 3000 membranes were independently scored by two investigators with very little variance between the scoring data. Table 3 provides a compilation of the scoring data, and Figure 5A−C provides examples of labeled membranes. In the untriggered condition, electron micrographs revealed a striking segregation of the labels: most of the membranes (84%) were labeled; however, only about 8% of the membranes were positive for both H^+,K^+-ATPase and biotin (Table 3 and Figure 5). The H^+,K^+-ATPase label was more prominent than the biotin label, which reflects the high density of the pump enzyme. When the membranes were triggered in our fusion conditions, the effect was dramatic: the number of membrane surfaces that clearly tested positive for both labels increased to almost 60% in the case of Mg^{2+}/ATP and 40% in the case of Ca^{2+} (Table 3), with a

Table 3: Localization of Gold Labels in Differentially Treated Tubulovesicles[a]

	no trigger		Mg^{2+}/ATP		Ca^{2+}	
condition	count	%	count	%	count	%
unlabeled	181	16.0	78	8.6	153	14.5
HK only	558	49.4	156	17.1	208	33.9
biotin only	302	26.8	132	14.5	100	11.1
both labels	88	7.8	544	59.8	297	40.5

[a] Two populations of tubulovesicles were labeled with different probes: one with a monoclonal antibody against H^+,K^+-ATPase, the other with biotinylated PE. Equal amounts of each membrane were coincubated at 37 °C for 5 min in the absence of fusion trigger or in the presence of fusion trigger: either 2 mM Mg^{2+}/ATP or 1 μM Ca^{2+}. Membranes were then laid onto nickel grids and stained with a combination of 15 nm gold-conjugated goat-antimouse IgG to detect H^+,K^+-ATPase and 10 nm gold-conjugated streptavidin to detect biotinylated PE. Membranes were then negative stained with phosphotungstic acid for EM observation and evaluation. A total of 2800 membranes were scored as to whether they had no label (unlabeled), 15 nm gold only (HK only), 10 nm gold only (biotin only), or both 15 and 10 nm gold (both labels). Data are shown for total membranes in each category (count), and the percentage (%) of each category are also shown for the three conditions.

corresponding decrement of both unlabeled membranes and singly labeled membranes. These results, taken with our previous data, indicate that our in vitro system reflects a genuine fusion event.

Introduction of Charges into Tubulovesicles with Ionic Detergents. We wondered whether the modifying effects of phospholipids were the result of specific recognition of the phospholipids by some enzymatic and/or signaling protein(s) or were due the effects of membrane charge, per se. To address this question, we introduced charges to the tubulovesicles by incubating them with ionic detergents at concentrations well below the critical micelle concentration for those detergents. These loaded membranes were then used as acceptors with untreated tubulovesicular donors. Use of the anionic detergent SDS produced striking effects in the fusion response. When loaded at concentrations between 0.01 and 0.5 mM, SDS caused marked increases in the triggered fusion response (Figure 6A), without causing spontaneous fusion or other modifications to the assay's baseline (data not shown). Similar results were obtained when monododecyl phosphate, another strong negative ionic detergent, was used (data not shown). On the other hand, when the cationic detergent DTAB was used, we observed the opposite effect: membrane fusion was suppressed (Figure 6B), with the effects of DTAB manifest at concentrations as low as 0.001 mM. These results support the assertion that the effects on fusion reported previously are due to charge, per se. It was of interest that we saw different results with the anionic detergents cholate (Figure 6C) and deoxycholate (Figure 6D), neither of which produced a significant effect on the fusion response, even at relatively high concentrations. While we do not have an explanation for this difference, these bile salt detergents are known to have less disruptive effects on functional activity of tubulovesicles. For example, when tubulovesicles are solubilized with SDS, the α- and β-subunits of H^+,K^+-ATPase dissociate; solubilization in either cholate or deoxycholate preserves this interaction.

If the net membrane charge on the loaded membrane is important for fusion, then we should be able to nullify SDS enhancement of membrane fusion by titrating the SDS with

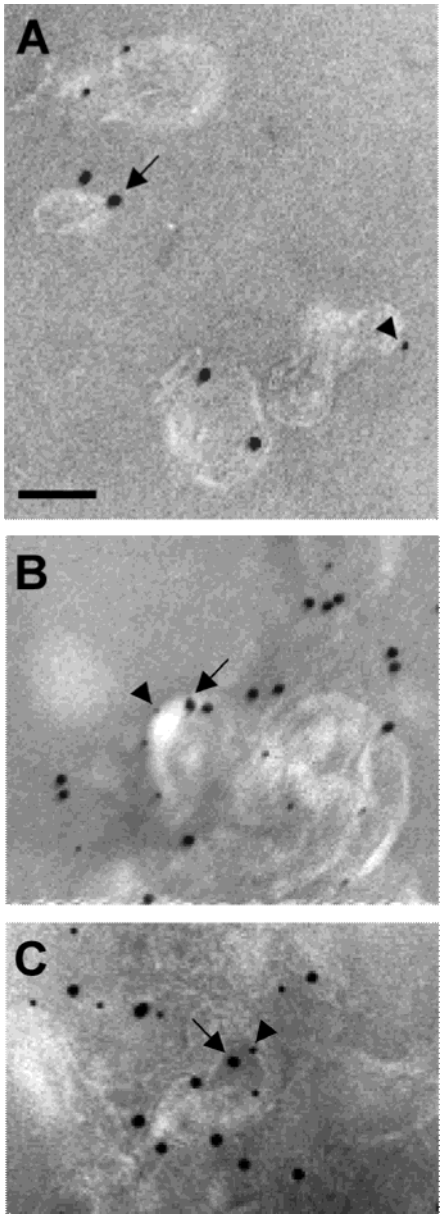

FIGURE 5: EM immunolabeling of tubulovesicles. Tubulovesicles were labeled with either 2G11 monoclonal antibody against H^+,K^+-ATPase or with phosphatidylethanolamine connected to biotin through a six carbon spacer (PE Biotin Cap) as described in the Materials and Methods. Membranes containing distinct labels were then mixed together and incubated for 3 min at 37 °C (A) in the absence of any trigger, (B) in the presence of 2 mM Mg^{2+}/ATP, or (C) in the presence of 1 μM Ca^{2+}. Membranes were then lain onto Formvar-coated nickel grids and treated with goat anti-mouse IgG conjugated to 15 nm gold and streptavidin conjugated to 10 nm gold. 2G11 labeled gold is indicated by arrows, and biotin label is indicated by arrowheads. In panels B and C, after the triggers were added, the labels are seen to colocalize on the same membrane.

DTAB. We took membranes preloaded with 0.05 mM SDS and loaded them with various amounts of DTAB. In accordance with our prediction, addition of DTAB reversed the stimulatory effect of preloading with SDS (Figure 7A). Membrane fusion was equal to that of untreated membranes when the amount of DTAB loaded was around 0.01 mM. This suggested that the net membrane charge was the determinant for enhancement of fusion, not the availability of charged sites on the membrane surface. We then asked whether a net negative charge on both populations of fusing

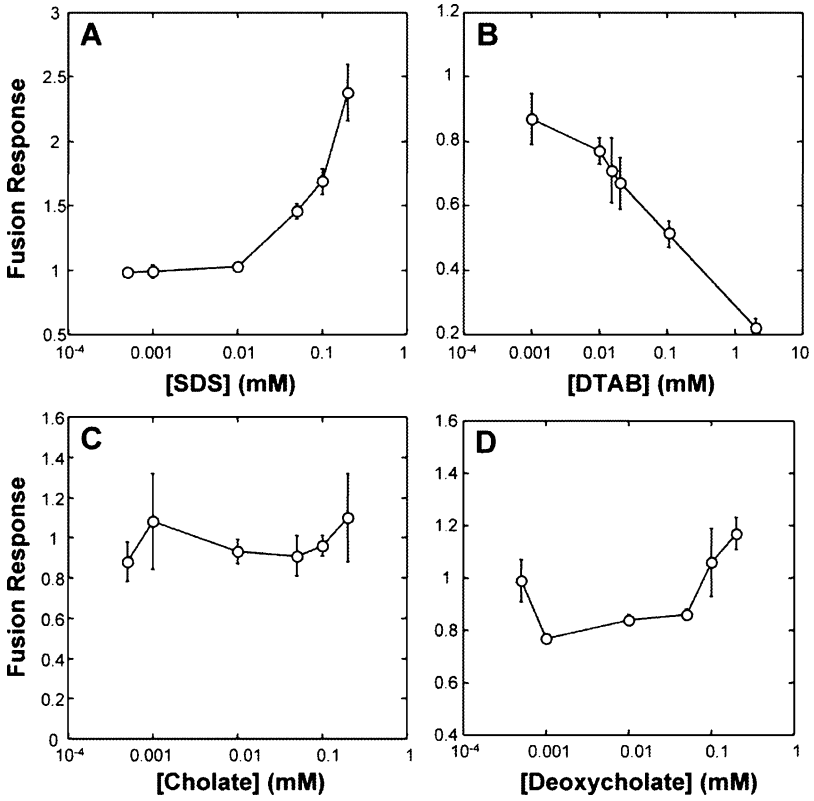

FIGURE 6: Effect of ionic detergents on tubulovesicle fusion. Tubulovesicles were loaded with the indicated concentrations of ionic detergents: SDS in panel A, DTAB in panel B, cholate in panel C, and deoxycholate in panel D. The loaded tubulovesicles were then used in fusion assays with unloaded tubulovesicle donors. Fusion response was calculated 2.5 min after triggering with 2 mM Mg^{2+}/ATP. A value of 1 represents the fusion response when unloaded tubulovesicles were used as acceptors. Data are shown ±SEM ($n = 3$).

membranes was conducive to fusion. As shown in Figure 7B, when we loaded acceptor membranes only with 0.05 mM SDS, we saw an increase in fusion that could be suppressed by also adding 0.01 mM DTAB to the acceptor membranes. When only the donors were loaded with SDS, we also saw an enhancement of membrane fusion. The fusion responses seen in the SDS-loaded donor and SDS-loaded acceptor experiments are not exactly the same; this can be explained by the fact that donors and acceptors are present at different concentrations in the assay (see Materials and Methods). Surprisingly, when both donors and acceptors were SDS loaded, we saw a further and significant increase in fusion response that could be reversed by loading either membrane with DTAB. When donor membranes were loaded with SDS and DTAB, they were less fusogenic than untreated membranes, even in the presence of SDS-loaded acceptors. These results indicate that a net negative charge on both fusing membranes, per se, greatly enhances the ability of gastric tubulovesicles to fuse with each other.

To test that the anionic effect is a surface charge phenomenon, we performed experiments in which we varied the ionic strength of the reaction medium. We normally perform fusion reactions in low ionic strength fusion buffer consisting of sucrose and only 5 mM HEPES buffer. The ability of a medium to shield surface charges increases with its ionic strength. Either NaCl or KCl was isotonically substituted for sucrose in the reaction medium, and the fusion was measured for the reaction between untreated tubulovesicle donors and tubulovesicle acceptors that had been loaded with 0.05 mM SDS. As shown in Figure 8, the fusion reaction decreased with increasing ionic strength for both the unloaded and the SDS-loaded membranes. Moreover, the two curves appear to converge; the difference at 140 mM NaCl, while significant, is markedly less than the difference at 0 mM NaCl. Separate experiments showed the same effect when KCl was used instead of NaCl (data not shown), suggesting that there is no specificity among monovalent cations. These results support the idea that negative charge acts on the fusion reaction via a surface potential effect, although they do not rule out other modes of action that may act in concert with the surface potential. Furthermore, they provide an explanation for the attenuating effects of ionic strength on the fusion reaction noted in an earlier study (*18*).

A possible artifact of the detergent loading experiments is that detergents might be solubilizing the membranes. To address this issue, we assessed solubilization by light scattering measurements. Tubulovesicles were suspended in SM at a concentration of 0.1 mg/mL and were mixed with incremental concentrations of detergent. Up to 1 mM SDS, there was little clarification of the membrane suspension, although raising the concentration of SDS to 2.5 mM significantly clarified the solution (data not shown). These results indicate that the detergent concentrations used in the loading experiments were not sufficient to solubilize the tubulovesicles. Light scattering data were also obtained using DTAB; under our working conditions, the membranes did not solubilize.

Activation Energy (E_a) of the Fusion Reaction. To further characterize the role played by negative surface charge, we measured the effects of temperature on the fusion reaction. Results shown in Figure 9A exhibit a strong temperature dependence for both control membranes and membranes loaded with 10 or 100 μM SDS, with the rates becoming very low near 5 °C. The temperature dependence was used

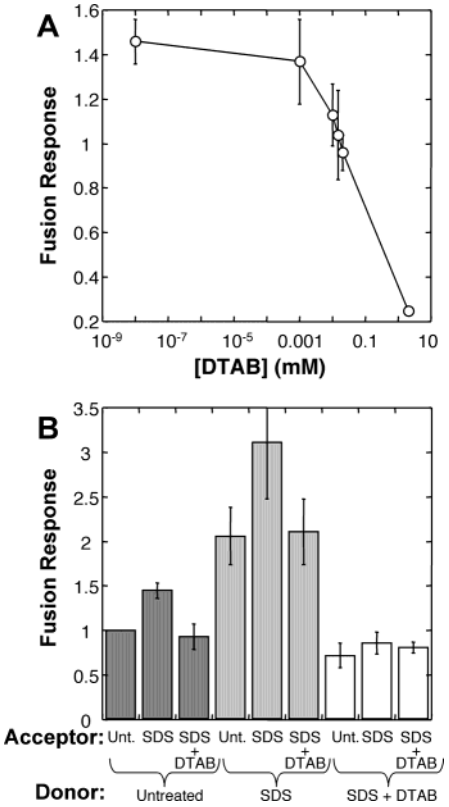

FIGURE 7: Anionic surface charge facilitates tubulovesicular fusion and is attenuated by cationic surface charge. (A) Tubulovesicles that had been loaded with 0.05 mM SDS were treated with increasing amounts of DTAB to titrate the anionic surface charge. These membranes were then used in fusion assays with untreated tubulovesicular donors. Fusion response was calculated 2.5 min after triggering with 2 mM Mg^{2+}/ATP. A value of 1 represents the value obtained when control, untreated acceptors were used; the SDS-loaded membranes had a mean response of 1.43-fold, with respect to control, prior to loading with DTAB. (B) Comparison of surface charge effects in donor and acceptor membranes. Tubulovesicles were loaded with 0.05 mM SDS (SDS), 0.05 mM SDS, and 0.02 mM DTAB (SDS + DTAB), or nothing at all (untreated). A portion of each of the unloaded and loaded membranes was labeled with R18 (donors) and mixed with acceptors in fusion assays triggered by 2 mM Mg^{2+}/ATP, as described above. All results are shown ±SEM ($n = 3$).

to estimate the activation energy (E_a) for the fusion reaction. To do this, we measured the first-order rate constants, k, for the Mg^{2+}/ATP-triggered fusion at several temperatures between 8 and 37 °C and used them to construct Arrhenius plots: ln k versus $1/T$, where T is degrees Kelvin. The slope of an Arrhenius plot is equal to $-(E_a/R)$, where R is the gas constant (eq 1). Figure 9B shows the temperature dependence of the fusion reaction rate for control tubulovesicles and tubulovesicles in which the acceptors were loaded with 100 μM SDS plotted in Arrhenius format. The data offer a reasonable straight line fit with a reduced slope for SDS-loaded membranes suggesting a decrease in E_a. Table 4 provides a summary of the mean E_a calculated from the measured temperature dependence of native tubulovesicle−tubulovesicle fusion rates as compared with rates when acceptors were loaded with 10 and 100 μM SDS. For the native membranes, the mean E_a was 14.1 ± 1.1 kcal/mol. Addition of 10 μM SDS produced a slight decrease in E_a (12.1 ± 0.7), which achieved the 5% level of significance with 100 μM SDS (11.0 ± 1.3 kcal/mol). The experimental data also suggested that a decrease in entropy of the fusion reaction was associated with addition of negative charge as the y-intercept of the Arrhenius plot ($x = 0$, proportional to change in entropy) was significantly decreased by SDS.

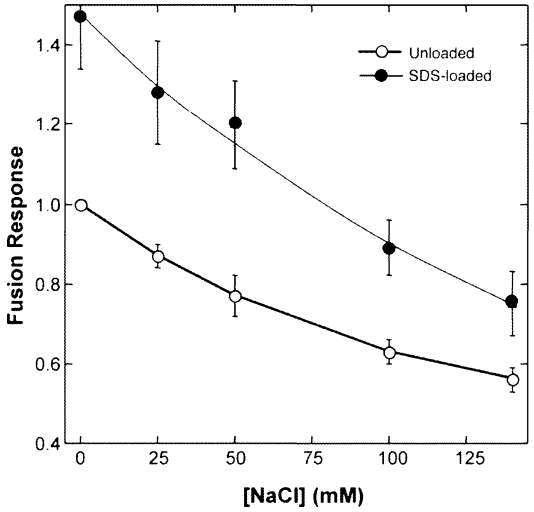

FIGURE 8: Titration with salt attenuates the tubulovesicle−tubulovesicle fusion reaction for normal membranes and those loaded with SDS. The 2 mM Mg^{2+}/ATP-triggered fusion reactions were carried out as described in the Materials and Methods using control tubulovesicles as donors and either control tubulovesicles (unloaded, open circles) or tubulovesicles loaded with 0.05 mM SDS (SDS-loaded, closed circles) as acceptors. The reaction media contained the indicated [NaCl] in the presence of 5 mM HEPES, pH 7.0. All solutions were isotonic with fusion buffer, with sucrose providing the osmotic balance where necessary. Control tubulovesicular acceptors used in the presence of 0 mM salt were assigned a fusion response of 1.0, and all other experiments were normalized accordingly. Replacing NaCl with KCl had no effect on the results (data not shown). Results are shown ±SEM ($n \geq 3$).

Table 4: Activation Energy of Fusion Reaction Is Decreased by Adding Negative Charge[a]

	E_a (kcal/mol)	y-intercept
control membranes (*9*)	14.1 ± 1.1	16.5 ± 1.8
10 μM SDS (5)	12.1 ± 0.7	12.7 ± 1.1
100 μM SDS (4)	11.0 ± 1.3[b]	11.9 ± 0.8[b]

[a] Data derived from initial rates of Mg^{2+}/ATP-mediated fusion of tubulovesicle donors and acceptors taken at a series of temperatures between 10 and 37 °C. Control, untreated membranes were compared with those in which the donor tubulovesicles were treated with either 10 μM SDS or 100 μM SDS as indicated. E_a was determined from the slope of the Arrhenius plot (similar to Figure 9B) for each membrane preparation as described by eq 1, and multiplied by $-R$, the gas constant, 1.987 cal/deg/mol. The intercept on the y-axis ($x = 0$) is the ln A term of eq 1 and is proportional to the entropy of the reaction. The number of membrane preparations tested is shown in parentheses. [b] $P < 0.05$.

DISCUSSION

The fusion machinery in gastric tubulovesicles is sensitive to the composition of lipid surfaces with which it fuses; this we have already established (*18*). In the present work, we sought to determine the basis for the preference. We show many data that illuminate the requirement for a negative charge in tubulovesicles or the membranes with which they undergo triggered fusion. There is almost no fusion reaction between tubulovesicles and pure DOPC liposomes. This is established by both the R18 assay and the protein transfer assay. When other lipids are incorporated into the DOPC mix, the most consistent and profound responses were to

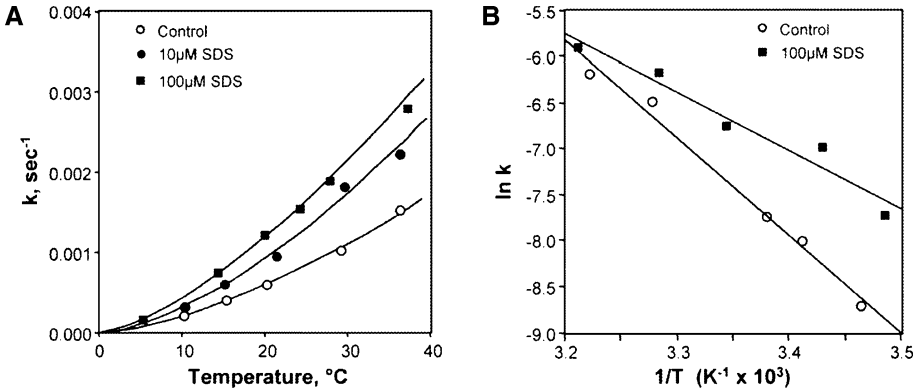

FIGURE 9: Temperature dependence of the fusion response for unloaded and SDS-loaded membranes. Control tubulovesicles (ctrl, open circles) and tubulovesicles that had been loaded with 10 μM (filled circles) or 100 μM SDS (filled squares) were used in fusion assays spanning temperatures from 5 to 37 °C. Detailed methods are described in the Materials and Methods. The exact temperature of each cuvette was measured immediately following a given fusion assay. (A) The relative initial fusion rates as a function of temperature (°C) for three separate experiments. Initial fusion rates were measured as the % change in total R18 fluorescence for the first 12 s after adding Mg^{2+}/ATP as trigger. Total R18 fluorescence was measured after solubilization of each sample with TX-100 after the temperature assays were completed. (B) Temperature dependence for Mg^{2+}/ATP-triggered fusion shown as an Arrhenius plot. The ln of initial rate data, such as shown in panel A, were plotted as a function of 1/*T*, converted to K deg^{-1}. The slopes of the lines (and their respective correlation coefficient) calculated by least squares were -10.6×10^3 s^{-1} deg^{-1} ($r^2 = 0.987$) for the fusion reaction of control membranes and -6.3×10^3 s^{-1} deg^{-1} ($r^2 = 0.951$) for the SDS-loaded membranes. A compilation of thermodynamic data is given in Table 4.

phospholipid with net negative charge. These reactions require a functional membrane protein in the biological membrane component, and they proceed only in the presence of one of the two major trigger conditions we used: Mg^{2+}/ATP or Ca^{2+}. The ability of negatively charged lipids to enhance the fusion response was reaffirmed by observations that tubulovesicles fuse preferentially with tubulovesicles that have enhanced negative surface potential. It makes little difference whether this surface charge is introduced in the form of phospholipids or through anionic detergents. On the other hand, the addition of positive charges tends to decrease the triggered fusion of tubulovesicles. We conclude that for the in vitro tubulovesicle fusion system studied here, anionic surface charge is essential.

A development in this work is that the ability to introduce exogenous lipids provided a confirmation assay for the triggered fusion process. Accordingly, we could introduce multiple membrane-trapped labels and evaluate the fusion process in the electron microscope, which may be of use to other fusion assays. We were able to score the labels by examining only clearly demarcated regions of lipid surfaces and directly visualize the mixing of intrinsic membrane components. It is clear that the striking changes in the membrane labeling before and after activation with either fusion trigger validate our fusion assays.

Experimental Considerations. We performed fusion reactions between tubulovesicles and tubulovesicles loaded with lipids to provide a more physiological setting to test a panel of lipids similar to those used in the liposomes. Two different methods were used: direct introduction of a chloroform solution and detergent solubilization followed by reconstitution (i.e., the OSGP method). Chloroform incubation proved to be generally very good for introducing lipid to the membrane. Recovery of both lipid and protein were excellent. Moreover, there was no apparent loss of membrane stability in the lipids loaded in this way, allaying fears that the organic solvent modified or permeabilized the membrane.

The OSGP/reconstitution method is a much more complicated way to add lipid to a membrane. Despite the great disruption of membranes that necessarily occurs during the course of solubilization/reconstitution, it has been successfully employed to functionally reconstitute many membrane transport proteins (*24*, *25*). The most common disadvantages noted were that the reformed membranes are highly variable in size and that recovery of protein can be low. We initially faced low protein recoveries but were able to optimize the procedure to recover >75% of the protein for any given reconstitution. As seen in Table 2, we were considerably less successful incorporating exogenous lipid using this technique. Despite these facts, our results were reproducible and quite similar to the results obtained using the chloroform incubation protocol. Moreover, the solubilization/reconstitution method promises to be extremely useful in future experiments to reconstitute the fusion response with protein components purified from the solubilized membranes. Issues do remain about both methods: for example, to which leaflet will a given phospholipid partition? In any case, it is clear that a negative charge facilitates fusion regardless of the technique that we used to introduce it.

Another issue with regard to our methods is our use of ionic detergents to manipulate the surface charge of membranes. Detergents cause leakiness and ultimately solubilize membranes. However, we have used concentrations well below those required to produce either of these effects on our membranes. Earlier work from our lab has carefully investigated the effects of many detergents on the ability of the membranes to generate and maintain proton gradients. We have kept our detergent concentrations well within the limits set by these studies.

Requirement of Negative Charge for Membrane Fusion. What, then, is the role of the anionic charge in membrane fusion? We believe that this can be explained, in part, by its role in defining the surface charge of the membrane. Both phospholipid bilayers and cell membranes can be induced to form transient pores by an electric field (*26*−*30*). This phenomenon has been widely used to introduce foreign substances, especially DNA, into cells. It has been proposed that a pore resulting from the electric field induced by close apposition of charged bilayers can comprise, or transit to, an intermediate state leading to membrane fusion (*31*). A

model such as this one is very interesting in light of the experiments in which we examined fusion between membranes that both possessed enhanced negative charge (Figure 7B). As both fusing membranes contained enhanced anionic surface charge, one may expect that a large electrostatic barrier has been erected between them, yet fusion is greatly enhanced. A theoretical model has been devised that describes membrane poration caused by the surface charge of cytosolic membrane leaflets in close apposition under physiological conditions that can lead to fusion (*32*). This mathematical model was based on exocytic cells of the bovine adrenal medulla, but the protein content and lipid composition of the membranes of interest (e.g., negatively charged PS is about 11% of total phospholipid) are similar to those of the rabbit gastric parietal cell (*14*, *33*). Also, the anionic phospholipid PS is confined almost exclusively to the cytosolic leaflets of membranes (*16*, *17*), an observation that teleologically supports the idea that surface charge of the membrane is important for fusion. The concept of membrane fusion proceeding through a pore-like intermediate, or fusion pore, is supported by many other observations using both biological membranes and artificial liposomes (*34*−*37*).

Before one acquires a commitment to the hypothesis that the surface charges of membranes are responsible for opening fusion pores, some obvious issues must be addressed. A consequence of this idea is that stable membranes would be unlikely to form in situations where two membranes are in close proximity, even within 100 Å of each other (*32*). Parietal cell cytosol contains many closely apposed, practically touching, membranes (*2*, *38*); one may suggest that if their electrical fields were able to instigate fusion, then none of these membranes would remain distinct. There are several reasons, however, why this difficulty is not insurmountable. First, the sensitivity of membranes to an electric field is greatly stabilized by their cholesterol content (*29*), and gastric tubulovesicles are relatively high in cholesterol (*14*). Second, the electric field would be shielded by cytosolic ions. Finally, the strength of the surface charge may not be a constant, and local effects may be more important than global ones. If the field effects were local and changeable, then the cell could increase the strength of the surface charge under appropriate conditions. Local effects on the negative surface charge of the cytosolic leaflet might be altered in vivo. Protein and lipid phosphorylation can increase surface charge, as can the generation of PA via normal phospholipid metabolism. PA generation is known to be required for fusion in neurons (*39*), mast cells (*40*), and PC12 cells (*41*). Moreover, the large increase in PA turnover observed with stimulation of gastric secretion suggests that PA metabolism may participate in parietal cell activation (*15*). While other roles have been proposed for PA at the fusion site (*40*), we suggest that at least part of its function is related to its effect on surface charge.

Another issue is that the apparent enhancement of fusion by negative charges might be a trivial effect caused by the positive charge of R18. For example, the fusion could simply be the consequence of the membranes sticking together because the added negative charge would increase, not decrease, the electrostatic attraction between the membranes. This is unlikely as protein transfer experiments demonstrate that triggered fusion occurs, and is enhanced by negative charge, in the complete absence of R18 (Figure 3). Moreover, fusion is enhanced by loading negative charge into either donor or acceptor membrane and is even more enhanced by loading negative charge into both membranes. Finally, to address the possibility that the presence of charged species in the membrane alters the R18 readout, we performed membrane titration experiments with R18 like those previously described (*18*). None of the lipids or detergents significantly affected the relative quantum yield of membrane-bound R18. Facing all of these data, we must conclude that the anionic effect is not an artifact caused by the use of R18.

Lessons from Other Fusion Models. The pioneering work of Papahadjopoulos and his group demonstrated an essential role for negatively charged lipids in the process of Ca^{2+}-induced fusion of synthetic liposomes. Their early studies (*42*) showed that Ca^{2+} in the range of 1−2 mM (or Mg^{2+} at slightly higher concentrations) promoted aggregation and subsequent fusion of liposomes made with, for example, 25% PS, balance PC, and/or PE. Inclusion of PA reduced the Ca^{2+} threshold (*43*), and several proteins were found to facilitate liposomal fusion and further reduce the level of Ca^{2+} required to trigger the reaction (*44*−*47*). Several interpretations have been put forward as a molecular basis to explain these liposomal fusion events. Fundamentally, the role of phospholipids in membrane fusion is related to their ability to form a dehydrated intermembrane complex at the point of contact. Addition of Ca^{2+} to liposomes that have a substantial negative surface charge would promote the complexation of interaction, possibly by forming a lattice region of alternating charge between apposing surfaces that exclude water and upset the stability of the surface thereby leading to fusion. Regardless of specific mechanism, the elimination of water is an essential step to promote the lipid flow into a fusion pore (*36*, *47*−*50*).

There have been long-standing arguments regarding the relevance of liposomal fusion toward interpreting fusion activity of native membranes. For instance, there are large differences in the free Ca^{2+} requirement, and the properties of specificity and vectorality can only be accomplished by the protein-rich biological membranes (*31*, *47*). Certainly the systems are different, but liposomal models have been useful in setting limits and allowing distinction between the local interfacial membrane association events and the subsequent phospholipid instabilities that must occur to promote fusion.

The work of Rothman and his group provides an exciting direction for describing the role of so-called SNARE proteins in determining the specificity of biological fusion events. They reconstituted specific SNARE proteins, or derivatives thereof, into phospholipid vesicles and measured the rates of fusion (*23*, *51*−*53*). Briefly, their results demonstrated that fusion occurred only when the appropriate cognate SNARE proteins were placed in the opposing membranes (i.e., when the vesicle SNARE protein known as VAMP-2 was reconstituted into one pool of liposomes, and the plasma membrane SNARE proteins known as syntaxin 1 and SNAP-25 were reconstituted in the other liposomal population). These data were taken to indicate that the SNARE proteins not only served as agents of recognition and specificity but also function as minimal fusion machinery.

Most models of membrane fusion recognize the membrane association event as a principal step in the process, and SNARE proteins with their tightly formed complex of

interaction provide excellent foci for association. However, additional factors, both lipidic and proteic, may be necessary to increase the probability for regional membrane destabilization and pore formation. For example, even when the reconstituted SNARE/liposome fusion rate was maximized by truncating discrete regions of individual SNARE proteins (*51*), the reported rates of membrane fusion were orders of magnitude slower than speeds required for in vivo fusion. Yet, in many of the experiments there appeared to be a very high percentage of effective SNARE protein complexation (*54*), which might suggest some missing elements in the machinery.

We propose that there are factors additional to the SNARE proteins that may promote lipid destabilization, thus greatly accelerating the directed fusion event. The present work suggests that the nature of the phospholipid headgroups may play an important role in the lattice of interaction between the adjacent interfaces. Moreover, these data also indicate that additional protein factors may facilitate the destabilization process so that the time constant for probability of fusion becomes closer to biological rates than observed in the SNARE reconstitution studies. We would argue that in our case these protein factors are triggered by Mg^{2+}/ATP or Ca^{2+}. It would be of interest to determine to what extent variations in specific liposomal phospholipids might contribute to the SNARE reconstitution studies, as well as other membrane proteins, perhaps utilizing energy from ATP.

Temperature Sensitivity of Membrane Fusion. The temperature sensitivity of ATP-triggered tubulovesicle fusion indicates a relatively large activation energy for the fusion response, which is not surprising considering the barriers inherent to membrane fusion. Introduction of negative charge into tubulovesicle acceptor membranes reduced the activation energy barrier for fusion and increased the fusion rate. Relatively high activation energies and high-temperature sensitivities, similar to those seen here, have been noted for the fusion responses of several biological and model membrane systems, including fusion pore formation in the mast cell granule secretion (*55*), the ATP-dependent step of chromaffin granule fusion/secretion (*56*), and fusion of protein free model lipid bilayers (*57*). Independent of whether one considers biological exocytosis, intracellular trafficking, formation of fertilization membrane, or enveloped viral infection, the biological fusion machine must ultimately facilitate the coalescence of membrane phospholipids. Indeed, based on the similarity of activation energy observed for secretory vesicle fusion, viral-mediated membrane fusion and liposomal membrane fusion, Lee and Lentz proposed that these fusion processes may involve some common lipid, rather than protein, rearrangements (*57*).

Is Surface Charge Sufficient? That surface charge plays a role in the fusion process is supported by much of the data presented here. Is surface charge adequate to explain our observations? The sensitivity of the fusion reaction to increasing ionic strength (Figure 8) is consistent with a contribution of surface charge, yet even at 140 mM NaCl, native tubulovesicles continue to fuse at more than 50% of their low-salt levels. Moreover, that the ionic strength curves of both unloaded and SDS-loaded tubulovesicles converge, but do not meet, at 140 mM NaCl may indicate that the negative charge has effects outside of a simple Gouy−Chapman surface charge model. One such effect may be to provide binding sites for proteins. Both VAMP (*58*) and synaptotagmin (*59*) possess negatively charged lipid-binding functionalities that are critical for membrane fusion in certain systems. Further, negatively charged membranes can affect the distribution of other SNARE molecules that do not have obvious lipid-binding domains (*60*). Another possibility is that the presence of negatively charged species might directly activate proteins in the membrane. PA, for instance, is known to activate phosphatidylinositol 4 phosphate kinase (*61*), and this role has been proposed to be critical in ARF-mediated exocytosis (*40*).

The basic thesis of this study, that negative surface charge is important, although not sufficient, for membrane fusion, is interesting in the light of conflicting ideas about whether biological membrane fusion is primarily a process of lipid biophysics or protein nanomechanics (*34*). Our data would seem to indicate that while the mechanism of fusion is largely dependent on the biophysics of phospholipids in an electric field, the kinetic control resides in other phenomena, probably protein nanomechanics. What roles do proteins play in this process? The most obvious role would be to force the membranes into close apposition; otherwise, the membranes would be more likely to repel each other instead of fusing with each other. Other roles could include controlling the opening and closing of the fusion pore once it forms and lending vectorality to the fusion process. SNARE proteins, which can form protein complexes between fusing membranes, and their associated proteins are obvious candidates to perform these roles. The biochemical properties of SNAREs clearly give them the ability to tie membranes tightly together. Because the SNARE complex is so stable, its formation may provide the energy required to force two membranes of like charge together. A wealth of evidence suggests that this may not be sufficient for membrane fusion in biological membranes (*62*−*68*), but it could clearly represent an important step thereof.

In considering the problem of membrane fusion from a lipid biophysics point of view, one must consider another important parameter, hydration of the headgroup. As the headgroups of membrane lipids are necessarily hydrophilic, and often charged to boot, a shell of water is associated with the membrane surface, representing a barrier to membrane fusion. Hydration of biological membranes is strong, in part because of their high PC content, as the choline headgroup is very strongly hydrated (*69*). How does the proposed mechanism for membrane fusion deal with this prohibitive barrier? Transient electroporation produced by an electric field was shown by ^{31}P NMR to produce massive rearrangements of polar headgroups within the affected membrane (*26*). These data were used to propose a model in which the rearrangement of headgroups weakens the hydration layer, making membrane reorganization, including poration and fusion, much more energetically favorable.

Finally, it is important to consider our results with regard to the action and metabolism of PI. PI can be phosphorylated on any of the five hydroxyl groups in its headgroup. This yields several different PI derivatives, each with a distinct function in cell signaling and membrane traffic (*70*). Virtually every inwardly directed membrane trafficking pathway in mammalian and yeast cells employs some PI phosphate, as does the biosynthetic pathway. Moreover, a central role for inositol phospholipid turnover has been reported for the Ca^{2+}-

activated, ATP-dependent, secretory pathway in adrenal chromaffin and PC12 cells (*71*, *72*). The effects of PI phosphates (PIPs) are generally mediated by proteins that bind to distinct PIPs through a number of PIP-specific binding domains (*70*). PIPs therefore provide a temporal and spatial scaffolding on which to build regulated proteic fusion machines. We expected marked changes in membrane fusion as a result of the addition of PIPs. The diminished sensitivity to PI seen in Figure 2C (as compared to PS and PA) may be the result of its net negative surface charge being shielded by the neutral inositol. As shown by Table 1, this is supported by further addition of phosphate to the inositol (i.e., PI_4P_2 and $PI_{4,5}P_3$), which enhance the fusion effects. On the other hand, there appears to be diminished fusion response to high concentrations of $PI_{4,5}P_3$. In any event, the effects that we observed PI phosphates did not exceed those that were mediated by PS or PA, suggesting that the negative charges per se were responsible for most of the effect. It remains possible that PIPs play a specific role in some aspect of parietal cell exocytosis but that our system does not reflect this because of the lack of appropriate cytosolic proteins.

CONCLUSIONS

Questions clearly remain about the biological relevance of these results. Of course, it was our initial goal to better understand the physiology of cells, particularly parietal cells. We have clearly shown that negative charge enhances the fusion of gastric membranes in vitro and that this can be reversed (back-titrated) by positive charge. Important questions remain as to whether and to what extent this is the case in the cell. While cell membranes can clearly alter their surface potentials in vivo through the actions of PI kinases and phospholipases, we cannot specify the degree to which this impacts the fusion process. Nor have we demonstrated that increasing the surface charge and enhancing exocytosis is even desirable in a physiological context. In some fusion processes, it is clear that docking of two membranes precedes, and is separate from, the actual fusion step (*63*, *67*). Why do these membranes not fuse immediately? It is important to realize that our thesis is not that negative surface charge is sufficient for fusion. For example, our previous studies demonstrate that functional protein is essential for fusion in this vitro system, and certainly regulators are required to trigger the process (*18*). However, we believe that by increasing negative surface charge in our system, we have observed some interesting results. We believe that these results possibly illuminate some feature of the membrane fusion apparatus that have not been properly appreciated.

In summary, the results of this paper indicate that negative membrane surface charge is required for fusion of gastric tubulovesicles triggered by Mg^{2+}/ATP or Ca^{2+}. This surface charge may perturb the lipid organization and structure in the fusing membranes by acting through an electric field at close proximity. It is unlikely that this is the only role played by the anionic charge, and we propose that it may also serve as a binding site and/or activator of certain enzymes and protein conformer states. Determining the roles of various proteins in this mechanism of fusion will be an important step toward testing this hypothesis and understanding the precise mechanism of tubulovesicular fusion. Considering the similarity of composition and functional activities of gastric tubulovesicles to various other membranes, it seems that this model may have more general applicability as well.

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BI036304Q