Fusion of Phospholipid Vesicles with a Planar Membrane Depends on the Membrane Permeability of the Solute Used to Create the Osmotic Pressure

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ABSTRACT Phospholipid vesicles fuse with a planar membrane when they are osmotically swollen. Channels in the vesicle membrane are required for swelling to occur when the vesicle-containing compartment is made hyperosmotic by adding a solute (termed an osmoticant). We have studied fusion using two different channels, porin, a highly permeable channel, and nystatin, a much less permeable channel. We report that an osmoticant's ability to support fusion (defined as the magnitude of osmotic gradient necessary to obtain sustained fusion) depends on both its permeability through lipid bilayer as well as its permeability through the channel by which it enters the vesicle interior. With porin as the channel, formamide requires an osmotic gradient about ten times that required with urea, which is ~1/40th as permeant as formamide through bare lipid membrane. When nystatin is the channel, however, fusion rates sustained by osmotic gradients of formamide are within a factor of two of those obtained with urea. Vesicles containing a porin-impermeant solute can be induced to swell and fuse with a planar membrane when the impermeant bathing the vesicles is replaced by an isosmotic quantity of a porin-permeant solute. With this method of swelling, formamide is as effective as urea in obtaining fusion. In addition, we report that binding of vesicles to the planar membrane does not make the contact region more permeable to the osmoticant than is bare lipid bilayer. In the companion paper, we quantitively account for the observation that the ability of a solute to promote fusion depends on its permeability properties and the method of swelling. We show that the intravesicular pressure developed drives fusion.

INTRODUCTION

Fusion between membranes is a widespread biological process. While the mechanisms responsible for fusion are unknown, it is clear that as a result of the process

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two phospholipid bilayer configurations must meld into one. It is now well established that osmotic swelling of phospholipid vesicles tightly adhered to planar bilayer membranes drives their fusion (Cohen et al., 1980; Zimmerberg et al., 1980; Cohen et al., 1982; Akabas et al., 1984; Woodbury, 1986; Niles and Cohen, 1987). Two procedures have been used to swell the vesicles. As illustrated in Fig. 1 *A*, the vesicles can be swollen by replacing an impermeant solute bathing the vesicles (*cis*

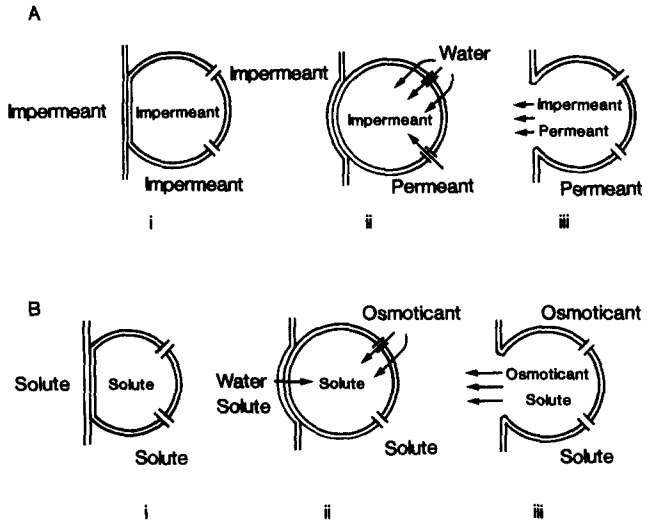

FIGURE 1. Methods for osmotic swelling of vesicles containing channels. In both *A* and *B*, the planar membrane separates two compartments with the vesicles added to the *cis* compartment. The vesicle shown contains channels embedded in its membrane; the channels allow permeation by water and some solutes. (*A*) Isosmotic substitution. (*i*) Initially, a solute that is impermeant through the channels is present at the same concentration in both compartments and in the vesicle. (*ii*) The channel-impermeant in the *cis* compartment is replaced with the same concentration of a channel-permeating solute. The permeant solute enters the vesicle through the channels, and water enters through both the channels and bare bilayer in the noncontact region. Water movement between the vesicle and the *trans* compartment is not depicted. (*iii*) Swelling results in fusion of the vesicle with the planar membrane. (*B*) Hyperosmotic swelling. (*i*) Initially, the solute is present at the same concentration in both compartments and the vesicle. (*ii*) An osmoticant is added to the *cis* compartment making it hyperosmotic. Both during and after an initial shrinkage, the osmoticant enters the vesicle through the channels and, possibly, through the bare bilayer. This creates an osmoticant gradient between the inside of the vesicle and the *trans* compartment which forces water into the vesicle. (*iii*) The vesicle swells and fuses with the planar membrane.

compartment) with an isosmotic quantity of a permeant solute (termed isosmotic substitution; Cohen et al., 1982). Both the diffusion of the permeant solute into the vesicles and the permeant solute's small reflection coefficient lead to water entry, thereby causing swelling and fusion. Swelling of vesicles can also be achieved by creating an osmotic gradient across the planar membrane that drives water into the vesicles adhering to the planar membrane (termed hyperosmotic swelling, see Fig.

1 *B*). The solution in the *cis* compartment is made hyperosmotic by adding a solute, termed an osmoticant. After an initial shrinkage, the ensuing flow of water from the *trans* to *cis* compartments and into the vesicles bound to the planar membrane causes their swelling and fusion. This method of swelling is routinely used to reconstitute ion channels into planar membranes via fusion (Moczydlowski et al., 1985). In a wide range of biological secretory systems, osmoticants have been added to cells to shrink granules, and fusion has been inhibited (Niles and Smith, 1982; Zimmerberg and Whitaker, 1985; Holz, 1986).

It has been noted in the model system that when the vesicular membranes contain porin channels, fusion could be obtained only with certain osmoticants, those that have relatively small permeabilities through lipid bilayer (Akabas et al., 1984). Fusion by hyperosmotic swelling of vesicles occurred with a 200 mosmol/kg gradient of salts, such as KCl, or nonelectrolytes, such as urea or glycerol. Attempts to obtain fusion with nonelectrolytic osmoticants having larger membrane permeabilities, such as formamide and ethylene glycol, were unsuccessful, even with osmotic gradients as large as 750 mosmol (Akabas et al., 1984).

In this paper, we report that fusion can be obtained with osmoticants possessing large membrane permeabilities, when the vesicles contain porin channels. However, osmotic gradients in excess of 2.0 osmol are necessary. Disparities between high and low permeability osmoticants are much smaller when nystatin is used as the channel in the vesicle. We also show that for the isosmotic substitution method of swelling, fusion is relatively insensitive to the lipid bilayer permeability of the solute substituted for the impermeant. Lastly, we find that the region of vesicle-membrane contact is not more permeable to osmoticants than unmodified lipid bilayer.

MATERIALS AND METHODS

Sonication-Freeze-Thaw Procedure

Porin was reconstituted into phospholipid vesicles to serve as a vesicular membrane marker according to the sonication-freeze-thaw procedure of Cohen et al. (1984) without modification. The lipid composition of the vesicles was 4:1 (wt:wt) egg phosphatidylcholine (PC): bovine phosphatidylserine (PS) (Avanti Polar Lipids, Birmingham, AL). The buffer consisted of 200 mM KCl, 10 mM HEPES, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.4.

Reconstitution of Porin in the Presence of Nonelectrolytes

To load porin-reconstituted vesicles with high concentrations of porin-impermeant nonelectrolytes, the freeze-thaw procedure could not be used because material precipitates by this procedure when nonelectrolytes are present (cf. Cohen et al., 1984). We therefore used a detergent-removal procedure. 5 mg of PC was dried in vacuo for 30 min. 0.5 ml of a clear solution of 100 mM octyl glucoside containing 25 μg of porin was added to the lipid mixture and the lipid was dissolved by swirling. This solution was applied to a Bio-Gel P-30 column (1 × 30 cm), which had been previously equilibrated with 5 mg of PC sonication-freeze-thaw vesicles devoid of porin. The vesicles were eluted with the column buffer, which consisted of 215 mM raffinose, 10 mM 2(*N*-morpholino)ethane sulfonic acid (MES), 2 mM $MgCl_2$, 0.1 mM EDTA, pH 6.0. Fractions were collected and their OD_{600}'s were determined in a spectrophotometer.

Large Vesicles

The fluorescent dye calcein was incorporated into the contents of large phospholipid vesicles by the procedure of Kim and Martin (1981) as described by Niles and Cohen (1987). The vesicle lipid mixture consisted of 2:1 (wt:wt) soybean lecithin type IIS (Sigma Chemical Co., St. Louis, MO):ergosterol. The vesicle contents consisted of 200 mM calcein, 10 mM MES, 5 mM *n*-propyl gallate, and 50 μg/ml nystatin, pH 6.0. The ergosterol was included in the vesicle membrane, and nystatin in its contents, so that the addition of nystatin to buffers bathing the vesicles would result in double-sided nystatin channels (Niles and Cohen, 1987). The vesicles were bathed in a buffer consisting of 650 mM sucrose, 10 mM HEPES, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.0.

Planar Membranes

Both "solvent-free" planar membranes formed by raising two phospholipid monolayers (Montal and Mueller, 1972) and solvent-containing black lipid membranes (BLM) were used. For the solvent-free membranes the lipid solution was 1% asolectin in hexane and for BLMs it was 5% asolectin in decane.

Detection of Fusion

Electrical detection of fusion was by measuring porin incorporation into voltage-clamped planar membranes as described by Cohen et al. (1984). Planar membranes were made in a circular orifice ~300 μm in diameter bored through the septum. Optical detection of dye release from vesicles fusing with the planar membrane was performed with a video fluorescence microscope as previously described in detail (Niles and Cohen, 1987). In brief, a fluorescence microscope with the stage removed was placed on its back brace. A Teflon chamber was mounted in place of the stage. A black Teflon septum with an orifice 160 μm in diameter divided the chamber into two compartments; the planar membrane was formed in the orifice. The front and rear walls of the chamber consisted of glass coverslips that enabled the fluorescent vesicles bound to the planar membrane to be imaged on the face of an SIT camera. The video signal was recorded on video tape for subsequent analysis. Vesicles were delivered to the planar membrane with an L-shaped pipette mounted to a micromanipulator, and connected to a nitrogen supply via an electric valve, which enabled vesicles to be ejected from the pipette at the planar membrane. The compartments of the chambers contained a small magnetic stirring bar to ensure complete mixing of the solution in the compartment.

Tracer Flux Measurements

Tracer flux measurements were used to determine the permeability of the planar membrane. [^{14}C]ethylene glycol (Amersham-Searle Corp., Arlington Heights, IL) was added to the *trans* compartment, and the *cis* compartment was sampled at periodic intervals.

RESULTS AND DISCUSSION

Osmotic Gradients across the Planar Membrane

It has been shown that fusion proceeds only if channels are present in the vesicles (Woodbury, 1986; Niles and Cohen, 1987). To investigate the effects on the fusion process of solute permeation through the bare bilayer and through the channels, we compared the efficacies of different solutes having differing permeabilities in porin-containing and nystatin-containing vesicles.

Porin vesicles. Fusion of porin-containing vesicles occurred routinely when 600 mM urea was added to the *cis* compartment (Akabas et al., 1984). But in scores of experiments, the addition of 750 mM of the more permeant osmoticants formamide or ethylene glycol did not result in any significant fusion. We have found that osmotic gradients of 2.0 osmol or greater are required for these permeant agents to promote fusion. This is shown in Fig. 2, where fusion of sonication-freeze-thaw vesicles containing porin channels with a solvent-free planar membrane is shown. When a 3.0 osmol gradient was established with formamide, fusion occurred. The rate was biphasic. The first phase was a burst due to fusion of vesicles that had accumulated

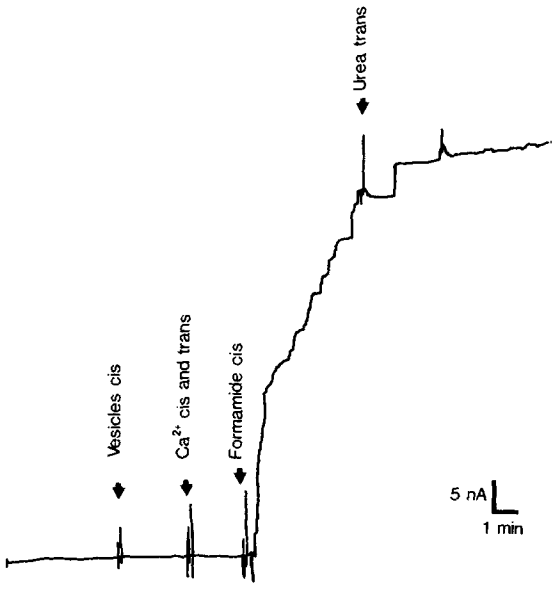

FIGURE 2. Fusion of porin-containing vesicles with a planar bilayer obtained with formamide. An asolectin "solvent-free" membrane, separating symmetric solutions (100 mM NaCl, 10 mM HEPES, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.0), was clamped at $V = +20$ mV. At the first arrow, 4:1 PC/PS sonication-freeze-thaw vesicles containing porin were added to the *cis* compartment to a concentration of $\sim 10^{11}$/ml. After several minutes, 25 mM $CaCl_2$ was added to both compartments. Subsequently, the *cis* compartment was brought to 3.0 M formamide by adding an aliquot of 12 M formamide in buffer. Fusion is manifested as stepwise increases in the voltage-clamp current. Each step is due to the simultaneous incorporation of each vesicle's porin channels into the planar membrane. After the addition of formamide there was an initial burst of fusion, due to previously bound vesicles, that lasted ~30 s. Thereafter there was a steady rate of fusion determined by the rate at which vesicles in solution bind and fuse to the membrane. When the transbilayer osmotic gradient was then decreased by adding 300 mM urea to the *trans* compartment, the rate of fusion was greatly decreased, indicating that a gradient of ~3 osmol formamide was necessary to obtain fusion of these porin-containing vesicles.

on the membrane after the addition of Ca^{2+} but before the addition of formamide. This was followed by the second phase, a steady rate of fusion due to vesicles from the *cis* compartment, which continued to bind and fuse to the planar membrane. This is similar to previously reported observations of fusion (Cohen et al., 1984). After the transmembrane osmotic gradient was reduced to 2.7 osmol by adding urea to the *trans* compartment, fusion was consistently slowed to a very low rate as illustrated in the figure. Similar results were obtained with BLMs with somewhat smaller gradients. Thus, when the vesicles contain porin channels, urea was about an order

of magnitude more effective an osmoticant than either formamide or ethylene glycol.

Nystatin vesicles. Swelling of vesicles containing nystatin channels was much less sensitive to the membrane permeability of the osmoticant than that of porin vesicles. Large vesicles (see Materials and Methods) were squirted, via pipettes, at planar membranes bathed by a sucrose buffer containing 20 mM $CaCl_2$ and 60 μg/ml nystatin. The efficacies of urea, formamide, and ethylene glycol as osmoticants in this system were evaluated by counting the number of vesicles that ruptured. Ruptures were detected as flashes of light (Niles and Cohen, 1987). No attempt was made to determine whether the dye was released to the *trans* compartment, as all ruptures were due to vesicle swelling (Niles and Cohen, 1987). The background rate of vesicle ruptures was first counted after ejecting vesicles at the planar membrane in the absence of an osmotic gradient (but in the presence of nystatin). Then 600 mM of either urea, formamide, or ethylene glycol was added to the *cis* compartment, and the rate of vesicle ruptures was determined.

Rates of fusion under ostensibly identical conditions are quantitatively variable when vesicles are added to the *cis* compartment (Cohen et al., 1984), a phenomenon well known to those who incorporate channels into planar membranes by fusion (Hanke, 1986). By directly ejecting vesicles toward the planar membrane in a controlled fashion the number of vesicles available to the planar membrane (which is one of the sources of variability) is better regulated. By determining the number of vesicular ruptures per squirt, variability, although still appreciable, is reduced and rates of fusion can be compared under different conditions. We determined "r," the average number of ruptures per squirt, for the three osmoticants by subtracting the background rate of ruptures from the total. With urea, r was 15.3 ± 11.5 (mean ± SD, $n = 6$ membranes), for formamide r was 11.5 ± 10.4 ($n = 4$), and for ethylene glycol r was 12.3 ± 12.5 ($n = 3$). As a control, in at least one experiment with each osmoticant, the osmotic gradient was then abolished by adding 600 mM of an osmoticant to the *trans* compartment and it was verified that flashing was blocked. In contrast to porin, it is clear that these three nonelectrolytes are comparably effective osmoticants with nystatin as the channel; because of the large variances any small differences between the osmoticants with nystatin would be difficult to detect. In contrast, with porin as the vesicular channel, at 600 mM only urea sustained fusion; formamide and ethylene glycol were totally ineffective at that concentration. Thus, an osmoticant's efficacy depends on the channels embedded in the vesicle membrane, which the solute must permeate to cause swelling.

Swelling without an Osmotic Gradient across the Planar Membrane

As swelling and the consequent development of a hydrostatic pressure is the driving force for fusion, the ineffectiveness of formamide and ethylene glycol as osmoticants with porin should be due merely to their inability to generate swelling. If a configuration were established that would allow these agents to promote swelling, then fusion should occur. That this is the case is illustrated by Fig. 3. Unilamellar vesicles, loaded with 215 mM raffinose and reconstituted with porin, were adsorbed to a bilayer bathed by 215 mM raffinose. The *cis* compartment was then perfused, substituting either 215 mM urea or 215 mM formamide for the raffinose. As raffi-

nose is relatively impermeant through porin channels (Nikaido and Rosenberg, 1981) (stachyose is totally impermeant, but unmercifully expensive), the urea or formamide entered the bound vesicles through the channels, and water followed; water also entered because of the small reflection coefficients of urea and formamide. As seen in the figure, formamide was effective in promoting fusion. A burst of fusion of bound vesicles was observed, followed by little or no fusion because vesicles were removed by perfusion. In contrast, for experiments of the type illustrated by Fig. 2 there was a constant supply of vesicles and steady rates of fusion were observed. Urea was perfused in to replace raffinose with 71 membranes. 53 of these experiments resulted in significant fusion and 18 did not. In 43 experiments, formamide replaced the raffinose; 20 of these led to significant fusion and 23 did not.

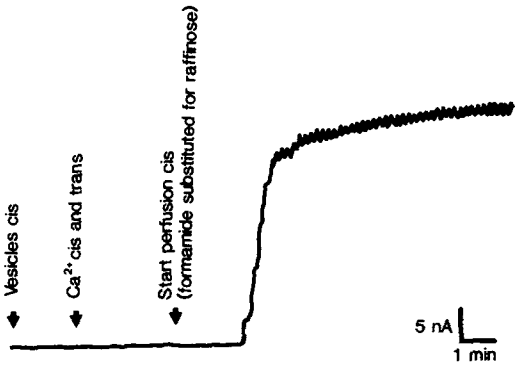

FIGURE 3. Formamide induces fusion when isosmotically substituted for a porin-impermeant solute. An asolectin BLM, clamped at V = +20 mM, separated two compartments containing 215 mM raffinose, which is relatively impermeant through porin channels. At the first arrow, 100 μl of PC vesicles reconstituted with porin and with 215 mM raffinose, were added to the *cis* compartment, which was stirred continuously throughout the experiment. 25 mM $CaCl_2$ was then added to both compartments. Subsequently, the 215 mM raffinose was perfused out and replaced with 215 mM formamide. After perfusing in the porin-permeant solute formamide, fusion was observed.

Region of Contact

We measured the ethylene glycol permeability of a planar bilayer adsorbed with vesicles to determine if the inability of this osmoticant to support fusion (of porin vesicles) at low osmotic gradients resulted from a large increase in the permeability of the bilayer in the region of contact between vesicles and planar membranes (Fig. 4). The flux of tracer amounts of [^{14}C]ethylene glycol was measured for a planar membrane. PC:PS (4:1) vesicles, reconstituted with porin, were then added to the *cis* solution, which contained 25 mM $CaCl_2$, and the flux measurement was continued. For this PC:PS mixture, Ca^{2+} did not cause aggregation of vesicles (Duzgunes et al., 1981). When hyperosmotic swelling was induced under these experimental conditions (but without the tracer), fusion proceeded. Vesicles therefore reached and adsorbed to the planar membrane. As seen, the permeability of the planar membrane was unaltered by the bound vesicles.

As the permeability of the planar membrane (and not the unstirred layers) deter-

mines the flux of ethylene glycol (Orbach and Finkelstein, 1980), the experiment above directly probes for large increases in bilayer permeability due to vesicle binding. At 25 mM $CaCl_2$, ~70% of asolectin vesicles that encountered the membrane were bound to it (Niles and Cohen, 1987), and nonaggregating vesicles can bind and completely cover a planar membrane (Akabas et al., 1984). If 10% of the planar membrane was in contact with vesicles, then an increase in permeability in the contact region by a factor of ten would have been easily detected as an increase, by a factor of two, in flux. Note that if the tracer entered the vesicle, it would exit relatively unimpeded into the *cis* compartment via porin channels. If the membrane in the region of contact were much more permeable, then for experiments in which urea was added to the *cis* compartment, urea would leak out of the vesicle at a higher rate and fusion would not occur (Niles et al., 1989). This fact combined with the above result leads to the conclusion that vesicle adsorption does not greatly increase the permeability of membranes in the region of contact.

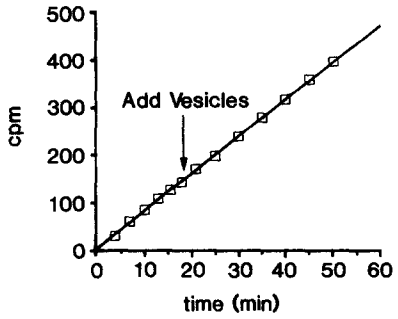

FIGURE 4. Permeability to ethylene glycol of an asolectin BLM unaffected by vesicle binding. The BLM was bathed by symmetrical solutions consisting of 100 mM KCl, 10 mM HEPES, 25 mM $CaCl_2$, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.4. [^{14}C]ethylene glycol was added to the 3-ml *trans* compartment to yield a specific activity of 20,000 cpm/50 μl. The *cis* compartment was sampled every 4 min; stirring of both compartments was maintained throughout the experiment. After 18 min, 50 μl of 4:1 PC/PS sonication-freeze-thaw vesicles were added to the *cis* compartment, and sampling continued. The addition of the vesicles had no effect on the transbilayer rate of ethylene glycol flux. The permeability coefficient (P_d) of this asolectin BLM to ethylene glycol was 9.1×10^{-5} cm/s.

Channels and Osmoticants

This paper shows that the potency of a solute in promoting fusion decreases as its permeability through the bare bilayer increases. The quantitative amount of the decrease depends on the method of swelling and the permeability characteristics of the channels in the vesicular membrane. Permeant osmoticants, such as formamide and ethylene glycol, are an order of magnitude less effective than relatively impermeant osmoticants, such as urea, when porin channels are used for hyperosmotic swelling. When nystatin is used, the potencies of the osmoticants in promoting fusion are comparable.

With isosmotic substitution, the permeant solutes are effective even when porin is the vesicular channel. In these experiments, there is no net removal of water from a corridor that might separate the vesicular and planar membrane in the contact region. Suggestions that osmotic gradients promote fusion, not by swelling the vesicles, but rather by removing water from this corridor (Fisher and Parker, 1984; Ehrenstein and Stanley, 1988), are inconsistent with the results of these experi-

ments. The experimental observations that channels are required for fusion and that the efficacy of a solute in promoting fusion depends on both the type of channel present and the mode of swelling are quantitatively explained in the companion paper (Niles et al., 1989).

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