

Fusion of Phospholipid Vesicles with Planar Phospholipid Bilayer Membranes

II. Incorporation of a Vesicular Membrane Marker into the Planar Membrane

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ABSTRACT Fusion of multilamellar phospholipid vesicles with planar phospholipid bilayer membranes was monitored by the rate of appearance in the planar membrane of an intrinsic membrane protein present in the vesicle membranes. An essential requirement for fusion is an osmotic gradient across the planar membrane, with the *cis* side (the side containing the vesicles) hyperosmotic to the opposite (*trans*) side; for substantial fusion rates, divalent cation must also be present on the *cis* side. Thus, the low fusion rates obtained with 100 mM excess glucose in the *cis* compartment are enhanced orders of magnitude by the addition of 5–10 mM CaCl₂ to the *cis* compartment. Conversely, the rapid fusion rates induced by 40 mM CaCl₂ in the *cis* compartment are completely suppressed when the osmotic gradient (created by the 40 mM CaCl₂) is abolished by addition of an equivalent amount of either CaCl₂, NaCl, urea, or glucose to the *trans* compartment. We propose that fusion occurs by the osmotic swelling of vesicles in contact with the planar membrane, with subsequent rupture of the vesicular and planar membranes in the region of contact. Divalent cations catalyze this process by increasing the frequency and duration of vesicle-planar membrane contact. We argue that essentially this same osmotic mechanism drives biological fusion processes, such as exocytosis. Our fusion procedure provides a general method for incorporating and reconstituting transport proteins into planar phospholipid bilayer membranes.

INTRODUCTION

The preceding paper (Zimmerberg et al., 1980) describes the fusion of phospholipid vesicles with planar phospholipid bilayer membranes as monitored by transfer of vesicular contents across the planar membranes (exocytosis). Although it provides a clear and convincing demonstration of fusion, this criterion is cumbersome and inconvenient for routine use. In this paper,

we describe another, more convenient criterion and consider some important parameters controlling fusion.

Our assay for fusion measures the appearance in the planar membrane of a membrane-associated marker which was present originally only in the vesicle membranes. The marker is the voltage-dependent anion channel (VDAC) obtained from mitochondria (Schein et al., 1976). This particular marker offers several advantages over those previously employed (Pohl et al., 1973; Cohen and Moronne, 1976; Moore, 1976): (a) VDAC is a naturally occurring, membrane-associated protein and is therefore unlikely, a priori, to be transferred independent of fusion, from one membrane to another. (b) Its conductance-voltage characteristic is unique and clearly distinguishable from nonspecific conductance changes that may occur in planar membranes (Schein et al., 1976). (c) We readily observe simultaneous (within 200 μ s) incorporation of several channels into the planar membrane; such events are unlikely to occur by independent transfer of channels from the vesicle membrane.

MATERIALS AND METHODS

VDAC-lipid mixtures were prepared by a modification of the method of Schein et al. (1976). Mitochondria were isolated from the livers of white Wistar rats (~200 g) by the method of Parsons et al. (1966). The mitochondria were osmotically lysed and washed twice in 1 mM KCl, 1 mM HEPES, pH 7.2, yielding a preparation of mitochondrial membranes. Excess lipid, 80% egg phosphatidylcholine (PC) + 20% bovine phosphatidylserine (PS) adjusted to pH 7.0 with KOH or HCl,¹ was added to a sample of these membranes to yield a final lipid-to-protein ratio = 25:1 (wt:wt). Protein was measured by the method of Lowry et al. (1951). This mixture was sonicated to clarity under N₂ at 4°C with the Cup Horn attachment of the Branson Sonifier, model W185 (Branson Sonic Power Co., Danbury, Conn.), at setting 10 (~140 W), lyophilized, and stored desiccated at -20°C. No loss of activity occurred over several weeks.

In some cases half of these mitochondrial membranes were treated (at a concentration of 1 mg/ml protein) with 1 M NaI for 30 min at 0°C, a procedure known to remove extrinsic proteins. This mixture was then centrifuged at 17,000 rpm (Sorvall SS 34 rotor) for 10 min. 30% of the protein remained with the membrane pellet. The pellet was washed three times in 1 mM KCl, 1 mM HEPES, pH 7.2. At this point excess lipid was added to both the original mitochondrial membranes and this purified membrane fraction at a constant lipid-to-protein ratio (25:1).

Multilamellar vesicles were prepared by dissolving 2.5 mg of the VDAC-lipid mixture in ~4 ml of hexane in a round bottom flask, drying the mixture by rotary evaporation at room temperature, adding 1 ml of buffer (100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0) along with three glass beads, and then shaking for a few minutes (Bangham et al., 1974). Vesicles were kept on ice and prepared fresh each day.

Planar membranes were formed at room temperature (22–24°C) by the brush technique of Mueller et al. (1963) across a 1-mm² hole in a Teflon partition separating two lucite compartments. Each compartment contained 3 ml of 100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0. The membrane-forming solution was either 5% asolectin (purified by the method of Kagawa and Racker [1971]), 4.5% of this asolectin + 0.5% diphytanoyl phosphatidylcholine (DPPC), or a mixture of 2% bovine PS +

¹ The pH of dispersed PS solutions was erratic and required adjustment with KOH or HCl.

0.5% egg PC (that is, 80% PS + 20% PC) in *n*-decane. After the membrane formed, approximately 10^9 vesicles (0.15 mg of lipid) were added to one compartment (henceforth called the *cis* compartment). In some experiments the vesicles were sonicated for 10 s in a cylindrical bath sonicator prior to addition.

All electrical measurements were done under voltage clamp conditions with a single pair of saturated calomel electrodes contacting the solutions via KCl junctions; the *cis* compartment was virtual ground. Either an Analog Devices 42L or a Teledyne Philbrick 100301 operational amplifier with a $10^8 \Omega$ feedback resistor was used for the voltage clamp; current was converted to voltage and measured by an oscilloscope and a chart recorder.

Incorporation of VDAC into the planar membrane is easily detected because of its unique characteristics. Steady-state conductance is maximal around $V = 0$ and falls rapidly for positive and negative voltages greater than ~ 20 mV (Schein et al., 1976). When the applied voltage is changed from $V < 10$ to $V = 40$ mV, the resulting current decays over several seconds from its initial high value to a lower steady-state value (Schein et al., 1976). With few membrane channels present, the closing of individual channels can be observed readily (Fig. 1).

DPPC was from Avanti Chemical Co. (Birmingham, Ala.). All other materials were as described in the previous paper (Zimmerberg et al., 1980).

RESULTS

The Basic Response

When multilamellar vesicles are added to one side of a planar membrane, no conductance increases are observed even after 1 hr. Upon subsequent addition

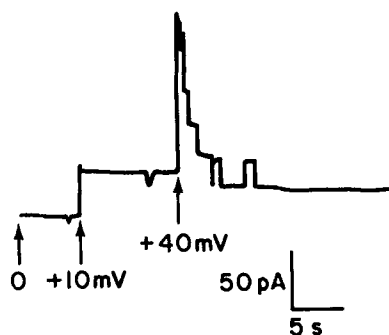


FIGURE 1. Voltage-dependent properties of VDAC. Five channels have been inserted into an asolectin membrane by adding a small amount of purified VDAC in Triton X-100 to the *cis* compartment. The current through the membrane is recorded as the voltage is clamped at different levels. To simulate the conditions of the fusion experiments, both compartments contain 100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0; the *cis* compartment in addition contains 40 mM CaCl_2 . Because VDAC is anion selective and a chloride gradient exists across the membrane, a current (30 pA) flows across the membrane from *trans* to *cis* at $V = 0$. At 10 mV the channels remain open. When V is changed to 40 mV, the instantaneous increase in current is three times that in going from $V = 0$ to $V = 10$ mV. The current then decays in steps, with characteristic kinetics, as the channels close one by one.

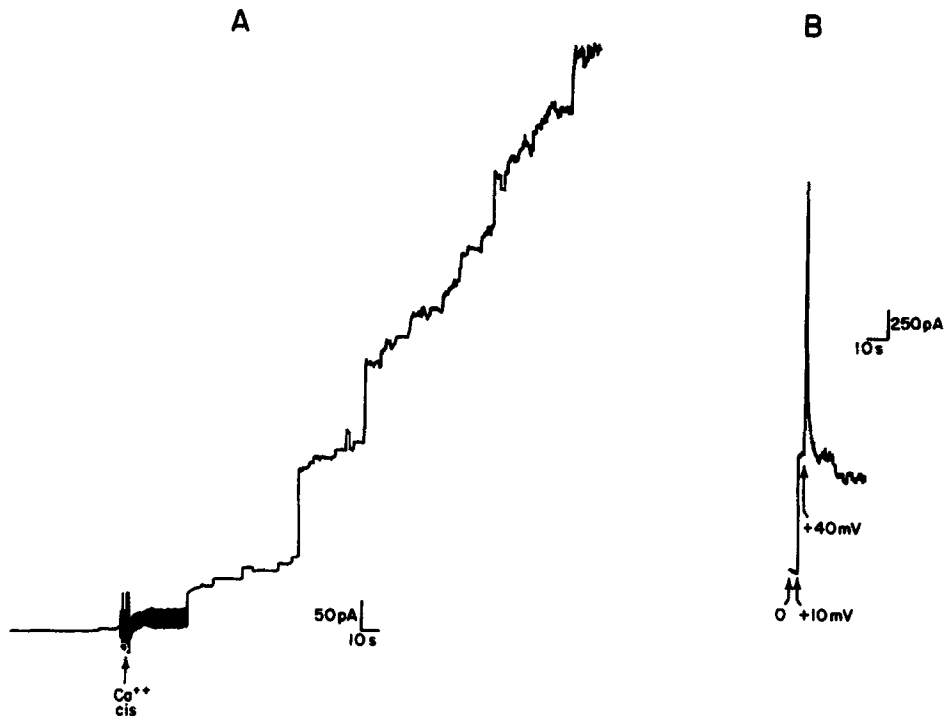


FIGURE 2. Fusion of VDAC-containing vesicles with a planar membrane. (A) A membrane (4.5% asolectin + 0.5% DPPC) clamped at $V = 10$ mV separates symmetrical solutions (100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0). 15 min prior to the record shown, vesicles were added to the *cis* compartment to a concentration of $\sim 3 \times 10^8/\text{cm}^3$. There was no increase in current during the following 15 min. At the arrow, CaCl_2 is added to the *cis* compartment to a concentration of 40 mM. After a lag of about 30 s, the current rises in discrete jumps over the next 10 min (only about 4 min of record are shown). Each discrete current jump corresponds to a fusion event. The variation in jump sizes reflects the heterogeneity of the vesicle preparation; i.e., it reflects the variability in the number of VDAC molecules in the outermost lamellae resulting both from the size dispersion of the vesicles and from the dispersion of VDAC population in vesicles of a given size. (B) To confirm that the current jumps result from VDAC, a voltage sequence of 0–10–40 mV was applied across the membrane 10 min after the jumps began. At $V = 0$ there is a *trans* to *cis* current of 850 pA; this is already indicative of VDAC. When the voltage is stepped from 10 to 40 mV, the current decays in a manner characteristic of VDAC turning off. (In going from 10 to 40 mV, the instantaneous increase in current was three times that in going from 0 to 10 mV. This was seen on the oscilloscope record but is not resolved on the chart recorder record shown.)

of 30 mM CaCl_2 to the same side, discrete jumps of current (with membrane clamped at 10 mV) occur within 1 min (Fig. 2 A); these continue for 5–10 min, after which the current does not rise further. If the membrane potential is now increased from 10 to 40 mV, the current increases proportionately² and

² Because of the salt gradient and the anion selectivity of VDAC-containing membranes,

then decays in a manner characteristic of VDAC turning off (Fig. 2 B). The magnitude of the current decay is that expected if all of the current rise observed at 10 mV resulted from our VDAC mixture. When the membrane potential is returned to 10 mV, the original current level reappears. In control experiments with multilamellar vesicles not containing VDAC, conductance increases are not observed even upon addition of CaCl_2 to 80 mM. These results, although suggestive of fusion of vesicles with planar membranes, might merely reflect independent transfer of channels from vesicular to planar membrane. The following two sections address this issue.

Simultaneous Incorporation of Several Channels

Because of their large size, many vesicles have more than one VDAC molecule in the outermost lamella.³ Simultaneous insertion of several channels into the planar membrane would provide strong evidence for fusion as the mechanism of channel incorporation, rather than fortuitous, simultaneous independent transfer of individual channels.

Experiments to examine this phenomenon were performed with few vesicles ($\sim 10^7/\text{ml}$) added to the *cis* side, since the frequency of putative fusion events is low, and hence the character of the individual current jumps can be analyzed. The result of a typical experiment is shown in Fig. 3. After addition of 30 mM CaCl_2 to the *cis* side, a current jump is observed. If the applied potential is increased from 10 to 40 mV, several individual channels are seen turning off. Thus, the number of VDAC molecules contributing to the original current jump is determined, which in this case is about six.⁴ The time resolution of our system defines "simultaneous" as $< 200 \mu\text{s}$.

If VDAC incorporation into the planar membrane occurs by fusion of vesicles with that membrane, current jump sizes would be proportional to the number of VDAC molecules in the outermost lamella of the vesicles. Mean current jump sizes were determined in parallel experiments using vesicles made with the partially purified protein preparation (NaI-treated) and vesicles made with the same protein preparation prior to purification. The mean jump sizes were about 2.5 times larger in the former instance.⁵ Since the NaI treatment presumably removes extrinsic proteins, the loss of 70% of total protein by this treatment should result in a threefold enrichment of VDAC in the vesicle preparation made with the remaining protein.

current flows across the membrane from the *trans* to the *cis* compartment at $V = 0$. The increase in current when V is shifted from 10 to 40 mV is three times that occurring when V is shifted from 0 to 10 mV.

³ Because the VDAC preparation is a crude protein mixture of which VDAC is a minor component, it is not possible to predict the number of channels in the outermost lamella simply from the protein-lipid ratio. A rough estimate of this number can be extrapolated from the channel density observed in planar bilayer membranes made as by Montal and Mueller (1972) from the VDAC-lipid mixture used to make the multilamellar vesicles. From this procedure we estimate that vesicles of 10 μm diameter have five channels in their outermost lamella.

⁴ Multiconductance states of VDAC make the exact number uncertain.

⁵ Determinations of mean jump sizes discriminate against the smaller ones, which get lost in the noise.

Comparison to "Exocytosis" Experiments

The data on incorporation of a membrane marker (VDAC) into the planar membrane are in agreement with those on transfer of vesicular contents across the planar membrane (Zimmerberg et al., 1980); the results are consistent for (a) the requirement for CaCl_2 (~ 30 mM), (b) the number of events, and (c) the duration of events for 20 min. This agreement was further confirmed in three experiments in which both incorporation of VDAC into the planar

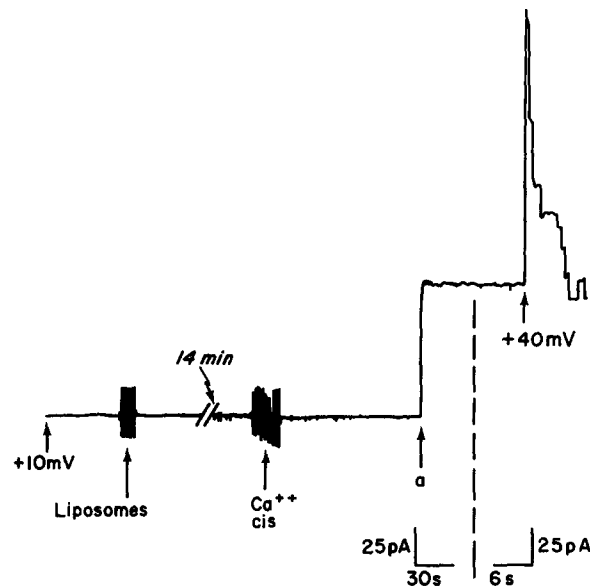


FIGURE 3. Demonstration of simultaneous insertion of several channels into a planar membrane by a single fusion event. A membrane (80% PS, 20% PC) clamped at $V = 10$ mV separates symmetrical solutions (100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0). VDAC-containing vesicles (liposomes) are added to the *cis* compartment ($\approx 10^7/\text{cm}^3$); no conductance increase occurs over the next 15 min. About 100 s after the addition of CaCl_2 to the *cis* compartment to a concentration of 30 mM, a single jump in current is observed (point *a*). When the clamping voltage is switched from 10 to 40 mV, several channels (about six) are seen turning off, thus demonstrating that the current jump at *a* resulted from the insertion of several channels.

membrane and appearance of vesicular contents in the *trans* compartment were measured (see Fig. 4 of Zimmerberg et al. [1980]). The number of fusion events recorded by the two methods agreed to within a factor of 2, with the incorporation method being the larger.⁶

⁶ This agreement is somewhat fortuitous, since there is no guarantee that the number of fusions of vesicles without VDAC in their outermost lamella is the same as the number of fusions of vesicles below the fluorimeter cutoff size (Zimmerberg et al., 1980). The former are not scored by the membrane marker assay, and the latter are not scored by the vesicular contents assay.

Effect of Osmotic Gradients

Fusion of vesicles with planar membranes is induced not only by CaCl_2 concentrations ≥ 30 mM but also by $\text{BaCl}_2 \geq 20$ mM and $\text{MgCl}_2 \geq 80$ mM. In all these instances, a significant osmotic gradient exists across the planar membrane in addition to the presence of divalent cation on the *cis* side. To what extent are these fusion events dependent on divalent cation and to what extent on the existence of an osmotic gradient across the planar membrane?

We have found that *both* divalent cations and an osmotic gradient are necessary to obtain significant numbers of fusion events; divalent cations alone are ineffective. Thus, fusion induced by 40 mM (104 mosM) CaCl_2 on the *cis* side is completely suppressed by abolishing the osmotic gradient across the planar membrane (Fig. 4), via addition to the *trans* side of 40 mM CaCl_2 , 60 mM NaCl, 120 mM urea, or 104 mM glucose. Reestablishing the osmotic gradient again results in fusion; experimentally this result is achieved either by removing the "blocking" osmoticant from the *trans* side (Fig. 5) or by adding more osmoticant to the *cis* side (Fig. 4). Clearly, an osmotic gradient, *cis* side hyperosmotic, is required across the planar membrane for vesicle-membrane fusion.⁷ (Fusion was never induced by making the *trans* compartment hyperosmotic with respect to the *cis*.) In fact, fusion is obtained with a 100 mM osmotic gradient created by urea or glucose alone, without divalent cation, although the frequency is low and is enhanced orders of magnitude by subsequent additions of Ca^{++} .

Thus, addition of 30 mM CaCl_2 to the *cis* compartment has a dual effect: the introduction of divalent cation and the creation of an osmotic gradient. These two actions can be separated. For example, significant fusion can be achieved with as little as 2–9 mM CaCl_2 in the *cis* compartment, provided that a large osmotic gradient is established by some other agent (e.g., glucose) (Fig. 6). More recently, we have obtained high fusion rates with 10 μM Ca^{++} in the presence of glucose-generated osmotic gradients, by inclusion of a calcium-binding protein in the planar membrane.⁸

DISCUSSION

Evidence That Vesicles Fuse with Planar Membranes

There are five major reasons for concluding that the effects described in this article result from fusion of phospholipid vesicles with planar phospholipid bilayer membranes: (a) The membrane marker, VDAC, is an integral membrane protein and hence on a priori grounds is unlikely to reach the planar membrane either by diffusion through the aqueous phase or by contact-initiated transfer from vesicle to planar membrane. (b) Simultaneous (<200

⁷ This is in contrast to vesicle-vesicle fusion (Miller et al., 1976) and sarcoplasmic reticulum vesicle-phospholipid bilayer membrane fusion (Miller and Racker, 1976), in which the vesicle contents must initially be hyperosmotic to the surrounding medium. In those instances, the osmotic effects are probably required to get the vesicles into a swollen, spherical state. During the time that fusion is occurring, no osmotic gradients exist, as the vesicles have long since reached osmotic equilibrium (due to water entry) with their surrounding medium.

⁸ Zimmerberg, J., F. S. Cohen, and A. Finkelstein. Manuscript in preparation.

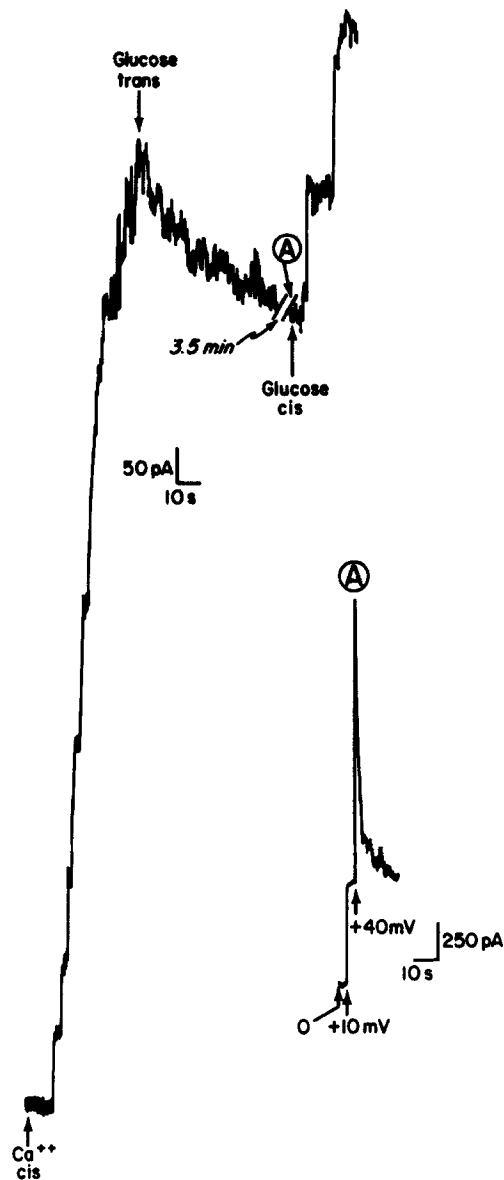


FIGURE 4. Demonstration that osmotic gradients affect fusion. A membrane (4.5% asolectin, 0.5% DPPC) separating symmetrical salt solutions (100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0) and clamped at $V = 10$ mV has VDAC-containing vesicles in the *cis* compartment at a concentration of $\sim 3 \times 10^8/\text{cm}^3$. Upon addition of 40 mM (104 mosM) CaCl_2 to the *cis* compartment, fusion proceeds. Addition of 104 mM glucose to the *trans* compartment immediately suppresses further fusion. (The small decline in conductance may represent inactivation of channels by decane.) Subsequent addition of 104 mM glucose to the *cis* compartment causes fusion to resume. The insert at (A) demonstrates that the conductance induced by fusion is characteristic of VDAC; i.e., it turns off when the voltage is shifted from 10 to 40 mV.

μs) insertions of up to 10 VDAC molecules argue strongly for a fusion mechanism, since processes mentioned in *a* would yield singlet insertions almost exclusively as in Fig. 7. Nor is it likely that VDAC is transferred as an aggregate from an already aggregated state in the vesicle membranes. For, with vesicles prepared using VDAC that is molecularly dispersed in Triton prior to formation of the vesicles, multiple, simultaneous insertions into the planar membrane still occur.⁹ (c) The number of channels simultaneously inserted into the planar membrane correlates with the number of VDAC

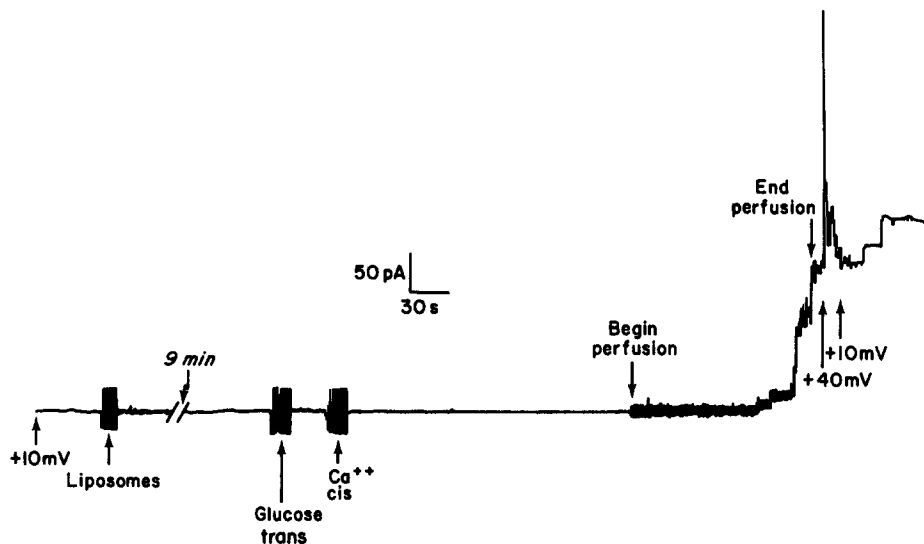


FIGURE 5. The effect of osmotic gradients on fusion. An asolectin membrane clamped at $V = 10$ mV separates symmetrical salt solutions (100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0). VDAC-containing vesicles (liposomes) are added to the *cis* compartment to a concentration of $\sim 3 \times 10^8/\text{cm}^3$. No fusion occurs during the next 10 min. Glucose is then added to the *trans* compartment to a concentration of 104 mM followed by addition of CaCl_2 to the *cis* compartment to a concentration of 40 mM (104 mosM). There is still no fusion over the next 7 min. When an osmotic gradient is established across the membrane by removing the glucose from the *trans* compartment (by perfusion with a glucose-free solution), fusion proceeds. Stepping the voltage from 10 to 40 mV demonstrates that the conductance induced by fusion is characteristic of VDAC.

molecules per vesicle. (d) A histogram of the number of channels inserted per event into the planar membrane correlates with the size distribution of the vesicles (Fig. 8), as expected for a fusion mechanism. (e) The agreement between the number and the time-course of events scored by marker incorporation (VDAC) into the planar membrane, on the one hand, and the

⁹ Direct additions of Triton-solubilized VDAC to the *cis* compartment result in only singlet insertions into the planar membrane (Fig. 7), and this process is not promoted by Ca^{++} .

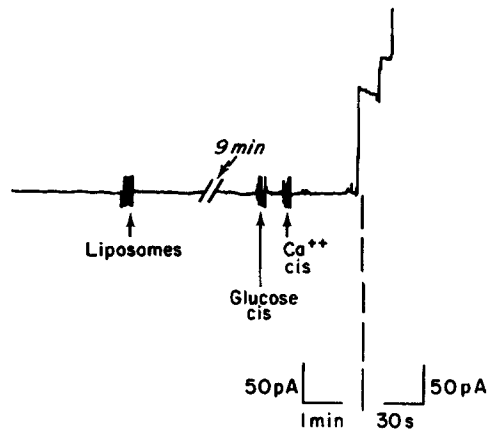


FIGURE 6. Demonstration that a smaller concentration of Ca^{++} promotes fusion when an osmotic gradient is established by another agent. An asolectin membrane clamped at 10 mV separates symmetrical salt solutions (100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0). VDAC-containing vesicles (liposomes) are added to the *cis* compartment to a concentration of $\sim 3 \times 10^8/\text{cm}^3$. No fusion occurs over the next 11 min. Following addition of glucose to the *cis* compartment to a concentration of 55 mM and CaCl_2 to the same compartment to a concentration of only 8 mM, fusion commences within 2 min.



FIGURE 7. Single-channel insertion of Triton-solubilized VDAC. An asolectin membrane clamped at $V = 10$ mV separates solutions containing 100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0, in both compartments and in addition 40 mM CaCl_2 in the *cis* compartment. Just prior to the start of the record, Triton-solubilized VDAC was added to the *cis* compartment (final Triton concentration = $3 \times 10^{-4}\%$). Current jumps corresponding to single insertions of VDAC occur, in contrast to the multiple insertions seen when VDAC-containing vesicles are added. Neither osmotic gradients nor Ca^{++} affect the insertion process of Triton-solubilized VDAC (not shown).

number and the time-course of events scored by the transfer of packaged fluorescent material across the planar membrane into the *trans* compartment, on the other hand (Zimmerberg et al., 1980), is weighty evidence for a fusion mechanism.

We conclude, therefore, that the events described in Results represent fusion of multilamellar vesicles with planar bilayer membranes. With $\sim 3 \times 10^8$ vesicles per ml in the *cis* compartment, there occur between 10 and 100 fusion events within 5–10 min.

Cessation of Fusion

We have consistently observed that fusion ceases, or its rate decreases drastically, ~ 10 min after the addition of ≥ 30 mM Ca^{++} to the *cis* compartment.

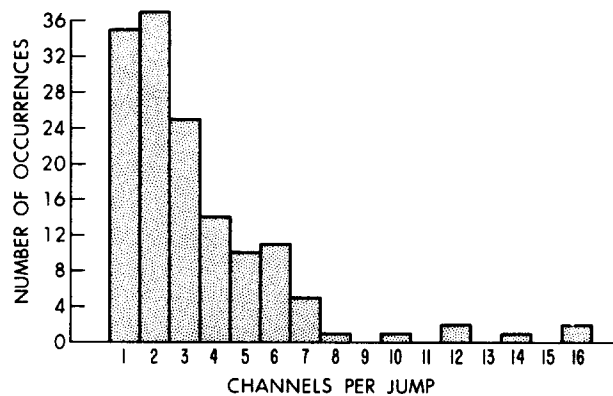


FIGURE 8. Histogram of the number of channels inserted into a planar membrane per fusion event. A membrane (80% PS, 20% PC) clamped at $V = 10$ mV separated symmetrical solutions (100 mM NaCl, 10 mM MES, 0.1 mM EDTA, pH 6.0). VDAC-containing vesicles were added to the *cis* compartment to a concentration of $\sim 4 \times 10^8/\text{cm}^3$. CaCl_2 was then added to the *cis* compartment to a concentration of 20 mM and current jump sizes were determined. The number of channels per jump was calculated from the known current jump size for a single channel (as determined from Triton-insertion experiments under the same ionic conditions). A total of 144 fusion events were counted. (The number of single channel jumps is underestimated, because as fusion proceeds, single insertions get lost in the noise.) Note that this histogram correlates with the size distribution of vesicles (see Fig. 2 of Zimmerberg et al. [1980]).

This is not because of changes in the planar membrane, since a new round of fusion can be produced either by addition of more vesicles to the *cis* compartment or by addition of 30 mM Ca^{++} to a fresh supply of vesicles in the *cis* compartment (following perfusion out of that compartment of the old vesicles and the Ca^{++}). We believe that the plateau in fusion activity results from Ca^{++} -induced aggregation of vesicles, which reduces the number available to fuse with the planar membrane. This interpretation is consistent with (a) the appearance with time in the *cis* compartment of clumped and precipitated

vesicles and (b) the longer times necessary to each a plateau when fusion is induced by smaller (~ 5 mM) Ca^{++} concentrations.

Mechanism of Fusion and Its Requirements

The two requirements for fusion of multilamellar vesicles with planar membranes are (a) a multivalent cation in the *cis* compartment and (b) an osmotic pressure difference (*cis* side higher) across the planar membrane. No fusion occurs if the first requirement alone is satisfied, and only very low fusion rates obtain if the second requirement alone is met. How do these two factors promote fusion?

That multivalent cations promote vesicle-vesicle fusion is well known. The resultant speculations of mechanism are equally applicable to the present case and include (a) electrostatic screening (or binding) of negative charges, which permits more frequent contact between vesicles and membrane (Lansman and Haynes, 1975); (b) bridging of charged groups between vesicle and planar membrane (Papahadjopoulos et al., 1978); (c) phase separation of lipids leading to fusion (Papahadjopoulos et al., 1977); and (d) local dehydration of charge groups on vesicle and planar membrane, which reduces a major energy barrier to contact (Hauser et al., 1975; Breisblatt and Ohki, 1976; LeNeveu et al., 1976). If electrostatic repulsion is the major barrier to fusion, the optimal choice of lipids should be ones with no net charge. Yet the smaller the percentage of negative charge in the planar membrane, the larger the Ca^{++} concentration required to promote fusion (Table I); no fusion was observed when the planar membrane contained no negatively charged lipids. Given the necessity, on whatever basis, for negative lipids, it is not unreasonable that multivalent cations promote vesicle-membrane contact by either binding or "bridging" charge groups, in addition to increasing the number of vesicles in the vicinity of the membrane by electrostatic screening.

The requirement for an osmotic gradient across the planar membrane was a surprising finding, which we believe is of major importance, and which we feel has broad applicability to fusion of biological membranes (see the following section). It is remarkable that vesicles fuse with planar bilayers in the presence of osmotic gradients in the complete absence of divalent cations,

TABLE I
EFFECT OF NEGATIVE CHARGE IN THE PLANAR MEMBRANE
ON THE AMOUNTS OF Ca^{++} REQUIRED TO PROMOTE
SIGNIFICANT RATES OF FUSION

Membrane-forming solution	$[\text{Ca}^{++}]$ required for significant fusion rates*
	<i>mM</i>
80% PS, 20% PC	2
50% PS, 50% PC	8
20% PS, 80% PC	20
5% PS, 95% PC	30

The lipid vesicles were 20% PS, 80% PC. In all experiments the sum of the glucose and CaCl_2 concentrations in the *cis* compartment made it hyperosmotic to the *trans* compartment by approximately 100 mosM.

* Greater than three per minute.

albeit at very low rates. This suggests that the osmotic pressure difference alone is the driving force for fusion, and that divalent cations simply decrease the activation energy barrier for this process. Thus, divalent cations are not strictly required for the fusion process but "merely" accelerate it.

In summary, we can view divalent cations as catalysts of a reaction driven by osmotic gradients. We envision a state of vesicle-planar membrane contact following collision of the vesicle with the membrane; divalent cations increase the lifetime of this state.¹⁰ Interestingly, the concentration of Ca^{++} required for fusion as a function of the percentage of negative charge in the membrane (Table I) indicates the necessity of a certain minimal amount of Ca^{++} at the membrane surface for catalysis of fusion, suggesting that Ca^{++} stabilizes vesicle-membrane contact by bridging charge groups (probably phosphate) between the two.

Osmotically induced water flow from the *trans* to *cis* compartment leads to fusion most probably by water entry into vesicles attached to the planar membrane; water entry into the vesicle results in bursting of the vesicular (and planar) membrane, at the region of vesicle-membrane contact, with subsequent discharge of vesicular contents into the *trans* compartment. An analysis of the flows and forces involved in this process is complicated by the multilamellar nature of the vesicles and the permeability of at least some, but probably not all, of the lamellae of VDAC-containing vesicles to the solutes in the *cis* compartment. A quantitative treatment must await experiments with single-walled vesicles; however, in the penultimate section of the Discussion we present some additional considerations concerning this process in single-walled vesicles.

Relation to Vesicle-Membrane Fusion (Exocytosis) Occurring in Biological Systems

The fusion of phospholipid vesicles with planar phospholipid bilayer membranes as described in this paper differs from biological vesicle-membrane fusion, as it occurs in such processes as transmitter release at chemical synapses or hormonal release by endocrine glands, in three major respects. Firstly, millimolar amounts of divalent cation (Mg^{++} , Ca^{++} , or Ba^{++}) are needed in the model system, whereas micromolar amounts specifically of Ca^{++} are required biologically (Baker and Knight, 1978; Baker and Whitaker, 1978). Secondly, an osmotic pressure difference is needed across the planar phospholipid bilayer membrane, whereas no significant osmotic pressure difference is apparent across presynaptic or glandular cell membranes. Thirdly, fusion rates are ostensibly much lower in the model system than in biological processes (such as stimulus-evoked synchronous discharge of transmitter at the neuromuscular junction). Superficially, then, the mechanism of phospholipid vesicle-phospholipid membrane fusion appears to bear little relation to the

¹⁰ This pre-fusion state is not irreversible. If it were irreversible, it would be possible, after first establishing the state in the absence of an osmotic gradient (40 mM Ca^{++} on both sides), to wash out unbound vesicles from the *cis* compartment (still maintaining 40 mM Ca^{++}) and to then obtain fusion from still-present bound vesicles by imposing an osmotic gradient (e.g., adding 100 mM glucose to the *cis* compartment). In fact, when this experiment was done, we did not obtain fusion.

mechanism(s) underlying biological exocytosis. We believe, however, that the differences are more apparent than real, and that, as discussed below, fundamental similarities exist between the fusion process we have studied and biologically occurring fusion.

CALCIUM SPECIFICITY A plausible role for Ca^{++} in exocytosis is to bring vesicles and plasma membranes into intimate contact. The small (micromolar) concentrations required specifically for Ca^{++} presumably reflect the presence of high affinity calcium-specific binding sites on the plasma membrane and/or the vesicle membrane. These sites are not present in phospholipid membranes; millimolar, rather than micromolar, amounts of (any) divalent cation are required to bind to phosphate and carboxyl groups in the two membranes (Hauser et al., 1975; Newton et al., 1978) and bring them into contact. Divalent cations may therefore function similarly in both the biological and the model system to bring vesicle and planar (plasma) membrane into contact. This viewpoint gains credence from our recent experiments,⁸ in which we effected fusion with micromolar amounts of Ca^{++} (even in the presence of millimolar amounts of Mg^{++}) when we incorporated into the planar membrane a calcium-specific binding protein ($K_m \approx 15 \mu\text{M}$ [Abood et al., 1976]).

OSMOTIC PRESSURE DIFFERENCE To effect fusion of phospholipid vesicles with planar phospholipid bilayer membranes, the solution on the *cis* (vesicle-containing) side of the planar membrane must be hyperosmotic with respect to the *trans* side. We postulate (see preceding subsection) that the entry of water into vesicles attached to the planar membrane bursts the vesicles and the planar membrane at the region of contact, causing vesicular contents to "pop" through to the *trans* side. Since no significant osmotic pressure gradients exist across plasma membranes in most animal cells, equivalent substitutes must apparently exist if this same mechanism pertains to biological fusion.

It is noteworthy, however, that the cytoplasm of fresh water protozoa is significantly hypertonic to the surrounding milieu (Prusch, 1977). (Contractile vacuoles maintain a steady state by continually pumping water out of the cell.) Mucocyst secretion by *Tetrahymena* (Satir et al., 1973) or trichocyst release by *Paramecium* (Satir and Oberg, 1978), which have been studied as prototypic examples of exocytosis, involve (presumably osmotic) entry of water into the mucocyst (or trichocyst-containing vesicle) attached to the plasma membrane; subsequent swelling and rupture of the vesicle then results in exocytotic expulsion of contents (Satir et al., 1973). Moreover, this process is suppressed when the tonicity of the surrounding medium is increased with sucrose (Satir, 1974). Thus, the osmotic swelling mechanism proposed for fusion of phospholipid vesicles with phospholipid membranes is directly applicable to mucocyst and trichocyst exocytosis in *Tetrahymena* and *Paramecium*.

For more traditional cases of exocytosis (e.g., hormonal secretion) in which significant osmotic pressure differences do not exist across the plasma membrane, several adaptations of the mechanism primitively manifested in *Tetrahymena* and *Paramecium* are possible: (a) The osmotically inactive contents of the vesicle (e.g., the catecholamine-ATP complex in chromaffin granules) might be mobilized to provide the required osmotic gradient. (b) The ionic

permeability of the vesicle membrane might increase, leading either to colloid osmotic swelling and rupture, or else to mobilization of inactive vesicular contents with subsequent osmotic swelling and bursting. This mechanism has been advocated by Pollard to explain epinephrine and protein release from chromaffin granules of the adrenal medulla (Pazoles and Pollard, 1978), serotonin secretion from platelets (Pollard et al., 1977), and parathyroid hormone release (Brown et al., 1978). In fact, the osmotic requirement for phospholipid vesicle-phospholipid membrane fusion is entirely consistent with the crucial role assigned by Pollard to osmotic lysis in exocytosis of large vesicles (as opposed to small, cholinergic vesicles). It is also consistent with a motion picture by W. W. Douglas showing that mast cell granules swell before release (quoted by Pollard et al., 1977). (c) If surface tension or elasticity is associated with the vesicular membrane, there is a higher hydrostatic pressure within the vesicle than in the cytoplasm. (The vesicular contents must then, of course, be hyperosmotic.) If the vesicle flattens at the region of contact with the plasma membrane, the pressure difference there, no longer balanced by surface forces, will burst the vesicle and plasma membrane, thus completing fusion. This mechanism has been suggested for catecholamine secretion from chromaffin granules (Smith and Winkler, 1972) but might be more appropriate to acetylcholine release at the neuromuscular junction, where surface forces can be more significant because of the small size of the vesicles.

We have focused above on the role of osmotic swelling in vesicle-membrane fusion (exocytosis). It should also be recognized, however, that this mechanism can apply equally well to other fusion phenomena such as cell-cell fusion. A large number of fusogenic agents (e.g., Sendai virus and lysolecithin) are also lytic agents (Poole et al., 1970; Fowler and Branton, 1977). This implies that they increase the permeability of cell membranes to, among other things, ions. Two cells in intimate contact, in the presence of these fusogenic agents, will undergo colloid osmotic swelling; rupture of the cell membranes at the region of contact will result in fusion. Indeed, Ahkong et al. (1973) conclude from their study of chemically induced fusion of hen erythrocytes that "... cell swelling by colloid osmosis plays an essential role in cell fusion."

FREQUENCY OF FUSION EVENTS The observed fusion rates appear rather low, considering that there are $\sim 3 \times 10^8$ vesicles per cm^3 in the *cis* compartment, and we obtain only ~ 100 fusion events in 10 min. This impression, however, is illusory. Assuming the mean diameter of fusing vesicles is about $1 \mu\text{m}$,¹¹ their diffusion constant (D) is $\sim 4 \times 10^{-9} \text{ cm}^2/\text{s}$ (calculated from the Stokes-Einstein equation). During the 10 min of fusion events (before vesicle clumping), vesicles diffuse a mean distance ($\sqrt{x^2}$) of $20 \mu\text{m}$ ¹²—which is less than the thickness of the unstirred layer on one side of the membrane (Holz and Finkelstein, 1970). The vesicles, therefore, that reach the 1-mm^2 membrane do so by diffusion and are contained in a $2 \times 10^{-5} \text{ cm}^3$ circular cylinder

¹¹ There is a large dispersion of vesicle size. The above calculation is very approximate and is offered only to show that the number of vesicles available for fusion is small. Any other reasonable choice of vesicle diameter leads to the same conclusion.

¹² Calculated from $x^2 = 2Dt$.

(base 1 mm^2 , length $20 \text{ }\mu\text{m}$). The total number, therefore, that can possibly fuse in 10 min is about 6×10^3 . Our observation of about 10^2 events means that $\sim 1\%$ of the vesicles that can fuse do fuse.

The probability of fusion is actually higher than this, for we consistently observe a much higher frequency of fusion during the 1st min after Ca^{++} addition than in subsequent minutes. Our interpretation is that vesicles in the immediate vicinity of the membrane fuse with a relatively high probability, and that the quasi steady-state rate is partially controlled by the rate at which vesicles diffuse into that region. This situation contrasts markedly to that at presynaptic terminals, such as neuromuscular junctions, where vesicles are densely packed about 50 \AA from the presynaptic membrane (Heuser, 1976). There diffusion is not rate limiting, and high fusion rates are possible. In summary, when correction is made for vesicle concentrations, fusion rates of phospholipid vesicles with planar phospholipid bilayer membranes compare favorably with those of cytoplasmic vesicles with plasma membranes. We also note that, physiologically, there is a low rate of secretion without stimulation (e.g., miniature end plate potentials [Fatt and Katz, 1952]¹³ and basal release of hormones [Palade, 1975]) and that increased intracellular Ca^{++} concentration following stimulation drastically increases this rate. This is analogous to the low fusion rates we observe in the absence of Ca^{++} (in the presence of an osmotic gradient) and the large increase in those rates when we add Ca^{++} on the *cis* side.

Shortcomings in the Model System

We have seen that the results from our model system are applicable to fusion phenomena that occur biologically. Two aspects of the model, however, are clearly not appropriate to biological phenomena. Although we feel these aspects are not essential to the fusion process, we address them here explicitly:

(a) Our model system involves fusion of multilamellar vesicles, rather than single-walled vesicles. If the multilamellar aspect of the vesicles is essential, then the phenomena we have described are not relevant to the more pertinent case of single-walled vesicles fusing with plasma membranes. Miller's observation, however, that incorporation of voltage-dependent channels from sar-

¹³ The frequency of miniature end plate potentials (mepp) increases when the medium is made hypertonic (Fatt and Katz, 1952). At first sight this effect appears to conflict with our theory of fusion, which predicts that the frequency should decrease when the medium is hyperosmotic with respect to the presynaptic cytoplasm. We believe, however, that the increase in mepp frequency in hypertonic solutions occurs under isoosmotic conditions; that is, the presynaptic terminal shrinks in response to the hypertonic solution and the cytoplasm quickly (within 1 s) becomes isoosmotic with respect to the medium. (The increase in mepp frequency under these conditions probably results from elevation of free cytoplasmic calcium concentration due to internal release of sequestered calcium [Shimoni et al., 1977].) We predict that if transmitter release could be measured immediately after the medium was made hypertonic (i.e., before the terminal had shrunk significantly and while there was still an osmotic gradient across the presynaptic membrane), transmitter release would be depressed. An appropriate test of this prediction would be to compare the quantal content of stimulus-evoked transmitter release in isotonic medium with the quantal content immediately after the medium was made hypertonic.

coplasmic reticulum vesicles into planar phospholipid bilayer membranes (which he believes occurs by fusion [Miller and Racker, 1976]) is dramatically promoted by osmotic gradients across the planar membrane¹⁴ indicates that our findings are applicable to single-walled vesicles. And recently, we have begun to obtain fusion of single-walled lipid vesicles with planar membranes. To obtain reasonable fusion rates, it may be necessary to pre-swell the vesicles in order for subsequent osmotic entry to lead to rupture, rather than merely to enlargement, of the vesicles.

(b) The planar membranes of the model system retain some decane (Fettiplace et al., 1971). Conceivably, the presence of hydrocarbon in the membranes is essential, and hence the phenomena we observed are not biologically relevant. We find, however, comparable fusion rates with planar membranes formed from phospholipids dissolved in hexadecane (rather than decane). Since planar membranes retain much less hexadecane than decane (Fettiplace et al., 1971), hydrocarbon is apparently not essential for fusion in our system. Definitive proof must await fusion of vesicles with hydrocarbon-free membranes.

Applications of Fusion

LIPID JUMP EXPERIMENTS When a protein is inserted into a planar membrane by the fusion technique, it is initially in the lipid milieu of the vesicle. With time, these lipids will exchange with those of the planar membrane, ultimately leaving the protein in a new lipid environment. This process can be followed, if the properties of the protein are affected by the surrounding lipid. For example, VDAC kinetics are much faster in stearylamine-containing membranes than in asolectin or PS-PC membranes. When PS-PC vesicles containing VDAC are fused with stearylamine-asolectin planar membranes, one can observe the relaxation of the VDAC kinetics from those of a PS-PC membrane to those of a stearylamine-containing membrane.¹⁵

ASSAY FOR MEMBRANE PURIFICATION At present, there is no convenient way of following the purification of a membrane channel. By incorporating channels into lipid vesicles at a known lipid-to-protein ratio, fusing them into planar membranes, and determining the mean number of channels incorporated per fusion event, one can readily follow the degree of channel purification. This is illustrated by our ability to monitor the partial purification of VDAC with NaI treatment.

RECONSTITUTION We have incorporated more VDAC into planar bilayers by the fusion process described in this paper than has otherwise proved possible. Since VDAC is a bona fide integral membrane protein, possibly other transport proteins also can be efficiently incorporated into planar bilayers by the same technique. We have already attempted fusion of natural vesicles (*Torpedo* microsacs, *Tetrahymena* cilia) using osmotic gradients and

¹⁴ Miller, C. Personal communication.

¹⁵ These observations were made for fusion events in which a very large number of channels were simultaneously inserted. Such events probably represent the fusion of large vesicles (> 20 μm diameter) with the planar membrane. The relaxation times of tens of seconds are consistent with the time required for the lipid in such a large inserted patch to exchange with the lipid in the rest of the membrane, assuming a diffusion coefficient of between 10^{-8} and 10^{-7} cm^2/s .

calcium and have obtained stepwise conductance increases. One can also hope to first reconstitute purified transport proteins in lipid vesicles, as has already been done (e.g., Racker et al., 1975), and then incorporate them into planar membranes via fusion. The requirement of millimolar concentrations of divalent cation could be a serious drawback in this endeavor, as these levels may be inhibitory or detrimental to transport function, particularly if present at the "inner" (i.e., cytoplasmic) surface of the protein. Our recent success⁸ in obtaining fusion with micromolar amounts of Ca^{++} is therefore very encouraging for the reconstitution possibilities with this system.

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