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PROTEIN-MEDIATED INTERMEMBRANE CONTACT FACILITATES FUSION OF LIPID VESICLES WITH PLANAR BILAYERS

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Fusion of phospholipid vesicles with planar bilayer membranes occurs provided there is an intermembrane contact, which can be mediated by phospholipid-binding proteins, even in the absence of calcium. The firm attachment phase is then followed by the osmotically-driven fusion. These results show that hydrophobic proteins (not necessarily Ca^{2+}-binding proteins) may enhance fusion by promoting contact of membranes. Such proteins may operate synergistically with Ca^{2+} to reduce the threshold concentration of Ca^{2+} needed for fusion of biological membranes. Protein-mediated intermembrane contact resulting in fusion may play a crucial role in the regulation and catalysis of biological fusion events.

Membrane fusion is a key event during the traffic and sorting of intracellular membranes. In the past, different models for fusion have been used in an attempt to elucidate its underlying molecular mechanism. For example, the role of Ca^{2+} has been extensively investigated using such model systems. Ca^{2+} enhances fusion of anionic phospholipid membranes [1,2]. The requirements for Ca^{2+} during fusion of lipid vesicles in model systems (i.e., millimolar concentrations) [1,2] are at least a few magnitudes higher than that required for fusion of cell-derived vesicles, which occurs in the micromolar range [3,4]. The higher threshold for Ca^{2+} observed in model lipid systems can be lowered to physiological levels by introducing Ca^{2+}-binding proteins into the membranes [5,6], a situation which would most likely be found in living cells. The enhanced sensitivity of Ca^{2+}-dependent fusion mediated by Ca^{2+}-binding proteins appears to be due to the close membrane contact induced by such proteins since lectin-induced intermembrane contact also enhances Ca^{2+}-dependent fusion of lipid vesicles [7].

Fusion of lipid vesicles with planar lipid bilayers, as a model system for fusion [2,8,9], is particularly attractive because of its simplicity, sensitivity, and apparent similarity to fusion of exocytotic vesicles with the plasma membrane. The transfer of a channel-forming protein from the vesicular membrane to the planar bilayer provides a sensitivity assay for fusion, allowing it to be monitored directly by means of a voltage-clamp set-up [2,8,9]. Using this approach, the conditions related to the fusion process can be investigated and dissected. In this system for example, the fusion of anionic phospholipid vesicles and planar bilayers requires the existence of an osmotic gradient across the planar membrane and the presence of Ca^{2+}. Presumably, the driving force for fusion is the osmotic swelling of the vesicles [9]. As pointed out, one possible explanation for the effect mediated by Ca^{2+} could be the increased intermembrane contact effected by Ca^{2+} (which would become more effective with a Ca^{2+}-binding pro-

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tein in the membrane [5,6]). If this assumption is correct, that is, if tight apposition of membranes is a prerequisite for osmotically-driven fusion, then, in principle, Ca^{2+} could be substituted by some other means of intermembrane contact. For instance, an intimate intermembrane apposition could be provided by phospholipid-binding proteins even in the absence of Ca^{2+} and should thus suffice in satisfying the requirements for fusion. This rationale leaves room for additional options in the control and selectivity of fusion of biological membranes, enlarging the repertoire of possible regulatory proteins which might modulate and direct fusion within the cell, some operating by means of simply mediating tight intermembrane contact. Here, we show that such protein-mediated contact of vesicles with planar bilayers, in Ca^{2+}-free conditions, is a sufficient condition for the osmotically-driven fusion to occur. We also demonstrate that this binding step precedes fusion and can be experimentally dissociated from the fusion process.

We use human plasma fibronectin as the phospholipid-binding protein. Fibronectin is a ubiquitous, high-molecular weight glycoprotein associated with cell surfaces [10]. It is thought to mediate a number of cell-surface phenomena, including that of cell-cell adhesion. It is known to bind strongly to phospholipids in the absence of divalent cations, to assume a different conformation in the presence of phospholipids, and to cause phospholipid vesicles to aggregate [11]. This binding effect appears to be specific for phospholipids rather than simply due to the result of hydrophobic interactions [11]. Fibronectin is now available in large homogeneous quantities.

As a marker for fusion, we use *Escherichia coli* porin, an outer membrane voltage-dependent channel that can be purified to homogeneity [12]. In earlier experiments we have used porin derived from *Neisseria gonorrhoeae* (the properties of which are described elsewhere [13]) and polymerized C9 of human complement, which also form channels (unpublished observations), all yielding similar results. Unilammelar vesicles containing porin can be prepared by the detergent-dialysis or detergent-dilution protocols [13–15] which yield membrane vesicle populations of very different sizes (40 and 400 nm, respectively, as examined under negative contrast electron microscopy). For reconstitution of porin, protein at 40 μg/ml is added to lipid suspensions containing detergent before dialysis or dilution. After formation of vesicles, as outlined elsewhere [13–15], the suspension is centrifuged at $100\,000 \times g$ for 90 min to pellet vesicles which are then resuspended to 1 ml of the same buffer. Porin-containing vesicles accumulate transiently [^{3}H]tetraphenylphosphonium$^+$ when equilibrated in equiosmolar sucrose [13,14], indicating functional reconstitution of a conductive pathway partially permselective for anions.

The bilayer is formed from monolayers of negatively-charged asolectin [16] by the technique of Montal and Mueller, as detailed elsewhere [13,14]. The potential of the *trans* side is defined as the virtual ground. Addition of porin-containing vesicles to one side (called the *cis* side) of a voltage-clamped planar bilayer causes little or no increase in membrane conductance (expressed in S, 1 S = ampere/volt), indicating that the baseline fusion frequency is very low (Fig. 1A). If Ca^{2+} (10 mM) is present, then setting up an osmotic gradient across the planar membrane, with the *cis* side hyperosmotic with respect to the *trans* side, leads to progressive fusion events which occur during the subsequent 15–20 min (Fig. 1A), a finding that is consistent with previously published data [2,9]. In the absence of Ca^{2+}, however, establishing an osmotic gradient does not result in any enhancement of fusion (fig. 1B). At this point, fusion can be catalyzed by the addition of fibronectin (50 μg/ml, 10^{-7} M) to the *cis* compartment (Fig. 1B). Fibronectin-induced fusion is efficient as evidenced by the immediate burst of fusion activity (which occurs even with 10^{-8} M fibronectin) but does not exhaust fusion of all fusion-competent vesicles since subsequent addition of Ca^{2+} (10 mM) promotes further fusion activity (Fig. 1B). The rate of fusion activity is proportional to the amount of added fibronectin and shows no saturation up to $3 \cdot 10^{-6}$ M of fibronectin (protein/lipid ratio of 1 : 100). The effect mediated by fibronectin does not depend on the size of the vesicle used; similar results are obtained with vesicle populations of different sizes, prepared as described above.

The effect of fibronectin is not restricted to negatively-charged lipids. Using a variety of other

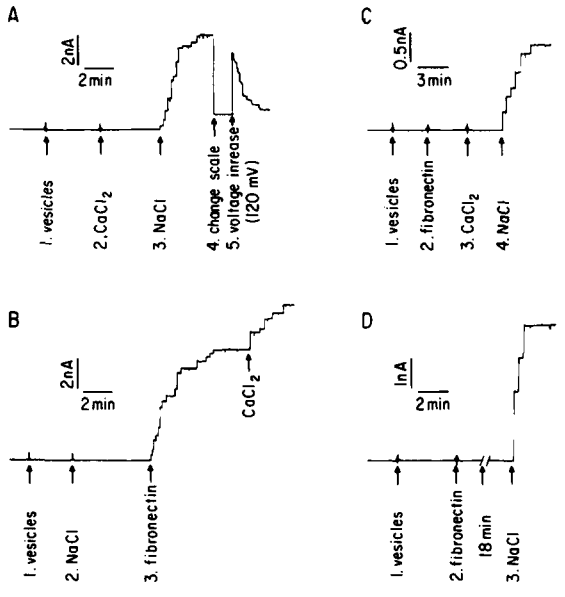

Fig. 1. (A) Effect of Ca^{2+} on osmotically-induced fusion of phospholipid vesicles with a planar bilayer membrane. The buffer bathing the membrane was symmetrical 0.1 M NaCl/10 mM Hepes (pH 7.0). The membrane was clamped at +30 mV throughout the experiments. The arrows point to the addition of various solutions (*cis* side, unless specified otherwise), followed by stirring of the chambers for 20–30 s. Addition of porin-containing vesicles to a final lipid concentration of 100 μg/ml (0.5 μg porin/ml) (indicated by (1)) did not result in any increase in baseline conductance. In (2) $CaCl_2$ was added to both sides to a concentration of 10 mM. No change of conductance occurred until an osmotic gradient was created across the planar bilayer by addition of NaCl to the *cis* side to a concentration of 200 mM (3). The current increase always occurred in step-wise fashion, indicating incorporation of single or groups of single channels into the bilayer. The current scale was changed in (4) (vertical bar, 10 nA) and the voltage stepped up from +30 mV to +120 mV (indicated by (5)). The immediate relaxation of current to a lower steady-state value corresponds to the voltage-dependent behavior characteristic of most bacterial porins indicating functional insertion of the porins into the bilayer. The order of addition of $CaCl_2$ and NaCl could be reversed, with qualitatively similar responses. (B) Effect of substitution of $CaCl_2$ by fibronectin on fusion. The bilayer was formed in 0.1 M NaCl/0.2 mM EGTA/10 mM Hepes (pH 7.0). (1) indicates addition of vesicles (100 μg lipid/ml) to the *cis* side. In (2) NaCl was added to the same side to 0.2 M. No current steps were observed, until purified fibronectin was added (indicated by (3)) to 50 μg/ml. After saturation of response, the addition of $CaCl_2$ (10 mM) produced another burst of fusion activity, indicating that fusion had not been exhausted at this point. Similar results were observed irrespective of the lipid used. Membranes formed with egg phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, diphytanolyphosphatidylcholine or monoolein responded similarly to an osmotic gradient. Fibronectin was purified as described [23], which under reducing conditions on SDS-polyacrylamide gels migrated as two closely spaced bands of M_r approx. 250000, consistent with the dimeric structure of this protein. (C) Synergistic effects of Ca^{2+} and fibronectin at low concentrations. Vesicles (100 μg lipid/ml) were added to a bilayer as indicated by (1), followed by fibronectin ((2) 5 μg/ml) and $CaCl_2$ ((3) 50 μM). With an osmotic gradient ((4) final concentration of 0.2 M NaCl on *cis* side), fusion steps occurred. The rate and magnitude of fusion attained under these conditions were at least 100-fold higher than those attained under the same levels of $CaCl_2$ or fibronectin tested separately. Membrane, buffer and vesicles were of the same composition as in (A). (D) Demonstration that binding precedes fusion. Membrane and buffer as in (A). In (1) vesicles were added, followed by fibronectin ((2) 50 μg/ml). 3 min later, the *cis* compartment was perfused with fresh buffer for 18 min (parallel bars) at 5 ml/min using a peristaltic pump. Following perfusion, NaCl was added to the same side to 0.2 M (3). This was followed by a burst of fusion activity.

defined lipids containing phospholipid moieties of different charges, similar effects are observed. However, with planar bilayers and vesicles prepared from phosphatidylethanolamine even in the absence of fibronectin and Ca^{2+}, a substantial amount of fusion activity is observed, which can be enhanced several fold following addition of fibronectin (not shown).

A combination of fibronectin and Ca^{2+} at low levels produces additive effects. The fusion activity that results from 10^{-8} M fibronectin and 50 μM Ca^{2+} is much higher than that produced by either one alone (Fig. 1C). This effect is specific for Ca^{2+}; addition of Mg^{2+} up to 1 mM does not enhance fibronectin-mediated effects.

Fibronectin binds vesicles to the planar bilayer. This inference can be demonstrated in experiments in which fibronectin is added to the *cis* side of a planar bilayer containing porin-containing vesicles, followed by perfusion of the same side with fresh buffer to remove unbound vesicles (Fig. 1D). If an osmotic gradient is then established across the planar membrane, a burst of fusion occurs, indicating that only vesicles tightly bound to the planar membrane and in the vicinity of the bilayer are subjected to the osmotic stress. This experiment also demonstrates clearly that a distinct pre-fusion binding step occurs as an intermediate of membrane fusion. This fibronectin-mediated binding step appears to be strong and irreversible; in the experiments in which the membrane is successfully perfused for at least 30 min, a burst of fusion

activity still occurs following the application of an osmotic gradient.

An additional piece of information consistent with the phospholipid-aggregation effect of fibronectin is that vesicles treated with fibronectin prior to addition to a planar bilayer lose the ability to fuse with the planar membrane subjected to an osmotic stress (data not shown). Vesicles which have been pre-treated with fibronectin form aggregates that can be seen under light and negative contrast electron microscopy (Ref. 11, and data not shown). The fusion-enhancing effect of fibronectin is evident only when it is added to the *cis* side of a planar bilayer already containing lipid vesicles.

A possible role for Ca^{2+} during fusion of biological membranes, in addition to the electrostatic effect (i.e., overcoming the repulsion forces of negatively-charged membranes), is the generation of an anhydrous state between the two lipid bilayers by displacement of water molecules from the lipid charge groups [1]. Similar regions of dehydration could be produced by hydrophobic and binding forces induced by lipid-binding proteins. Such forces could range from tight binding of protein-lipid molecules resulting in lipid vesicle aggregation, as in the case of fibronectin, to complete fusogenic activity, as evidenced with certain viral glycoproteins responsible for viral envelope fusion with cells. In regard to the latter, it may be interesting to point out that hydrophobic domains along viral fusogenic proteins become exposed under conditions which predispose to fusion, resulting in the attachment of viruses to membranes [17–19]. In fact, preliminary experiments show that purified viral fusogens may also be used in place of fibronectin to enhance lipid vesicle fusion with planar bilayers. The two types of forces (those mediated by proteins and Ca^{2+}) may work synergistically. A close intermembrane contact mediated by protein could enhance the specificity of subsequent binding to Ca^{2+}; indeed, changes in Ca^{2+}-phospholipid binding curves have been observed with intermembrane contact [20]. These observations would also imply that the protein-mediated enhancement of Ca^{2+}-dependent membrane fusion is not restricted to Ca^{2+}-binding proteins alone; lipid-binding proteins that can mediate tight intermembrane contact in general are suitable candidates for regulating intercellular fusion as well. The presence of such intracellular proteins could greatly increase the plasticity and sensitivity of the fusion events to intracellular regulatory signals. There is also need to investigate the role of other factors in fusion, as for example, the role of low pH in the fusion of endosomes and lysosomes. It seems likely that irrespective of the type of fusion observed in the cell, the tight intermembrane contact, which can be observed here (as mediated by fibronectin) and elsewhere (Ref. 9 Ca^{2+}-dependent), is a common and important intermediate.

What results in the completion of fusion of biological membranes after their close attachment remains unclear. There is some evidence for osmotic swelling of vesicles during fusion of biological membranes (reviewed in Ref. 21), a situation most easily observed during exocytosis. If osmotic swelling is indeed the driving force for fusion of exocytotic vesicles with plasma membrane, then it is conceivable that pore-formation in the vesicular membrane may be the molecular event immediately preceding fusion (which has also been suggested for electric field-induced fusion, [22]), and the sequence of steps could then be visualized as (1) tight attachment of membranes, (2) osmotic swelling of vesicle, (3) pore-formation, and (4) fusion. Although for the moment the relevance of these steps to fusion of biological membranes remains in the speculative plane, it is nevertheless exciting to note that experiments which will help dissociate the mechanism of fusion into well-defined steps may now be designed.

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References

1 Wilschut, J., Düzgünes, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021

2 Cohen, F.S., ZImmerberg, J. and Finkelstein, A. (1980) J. Gen. Physiol. 75, 251–270

3 Ekerdt, R., Dahl, G. and Gratzl, M. (1981) Biochim. Biophys. Acta 646, 10–22
4 Baker, P.F. and Whitaker, M.J. (1978) Nature 276, 513–515
5 Zimmerberg, J., Cohen, F.S. and Finkelstein, A. (1980) Science 210, 906–908
6 Hong, K., Düzgünes, N., Ekerdt, R. and Papahadjopoulos, D. (1982) Proc. Natl. Acad. Sci. USA 79, 4642–4644
7 Sundler, R. and Wijkander, J. (1983) Biochim. Biophys. Acta 730, 391–314
8 Miller, C. and Racker, E. (1976) J. Membrane Biol. 30, 283–300
9 Cohen, F.S., Akabas, M.H. and Finkelstein, A. (1982) Science 217, 458–460
10 Yamada, K.M. and Olden, K. 91978) Nature 275, 179–184
11 Rossi, J.D. and Wallace, B.A. (1983) J. Biol. Chem. 258, 3327–3331
12 Tokunaga, H., Tokunaga, M. and Nakae, T. (1981) J. Biol. Chem. 256, 8024–8029
13 Young, J.D.-E., Blake, M., Mauro, A. and Cohn, Z.A. (1983) Proc. Natl. Acad. Sci. USA 80, 3831–3835
14 Young, J.D.-E., Young, T.M., Lu, L.P., Unkeless, J.C. and Cohn, Z.A. (1982) J. Exp. Med. 156, 1677–1690
15 Young, J.D.-E., Unkeless, J.C., Young, T.M., Mauro, A. and Cohn, Z.A. (1983) Nature 306, 186–189
16 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487
17 Hsu, M.-C., Scheid, A. and Choppin, P.W. (1981) J. Biol. Chem. 256, 3557–3563
18 Wilson, I.A., Skehel, J.J. and Wiley, D.C. (1981) Nature 289, 366–373
19 Young, J.D.-E., Young, G.P.H., Cohn, Z.A. and Lenard, J. (1983) Virology 128, 186–194
20 Ekerdt, R. and Papahadjopoulos, D. (1982) Proc. Natl. Acad. Sci. USA 79, 2273–2277
21 Pollard, H.B., Creutz, C.E., Fowler, V., Scott, J. and Pazoles, C.J. (1981) Cold Spring Harbor Symp. Quant. Biol. 46, 819–834
22 Zimmermann, U. and Vienken, J. (1982) J. Membrane Biol. 67, 165–182
23 Furie, M.B. and Rifkin, D.B. (1980) J. Biol. Chem. 255, 3134–3140