The mechanics of biological membrane fusion

Merger of aspects from electron microscopy and patch-clamp analysis

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The problem and the methods available

Fast freezing (cryofixation) combined with freeze-fracture electron microscopy has shown that biological membrane fusion is a locally restricted and fast event (Plattner, 1981, 1991). It is difficult to capture, since it is at the limits of the temporal (0.1 to 1 ms, depending on conditions, like sample geometry etc.) and spatial (~3 nm) resolution available with this methodology (Plattner and Bachmann, 1982). It has been established that some fusion sites contain intramembrane particles (IMPs, equivalent to integral proteins) and a diffuse mass of proteins between membranes to be fused (Plattner, 1981, 1991). Later on, patch-clamp analysis of membrane capacitance changes (Neher and Marty, 1982) has not only refined the concept of fast focal fusion, but recently has also provided a new model of the possible role of integral membrane proteins in fusion induction (Almers, 1990). A number of biomembrane fusion processes have been analyzed by both methods, each one having its specific capabilities (Table 1). Current attempts aim at identifying molecular equivalents of these aspects. Only a combination of the different approaches can finally solve the problem of biomembrane fusion.

Analysis of membrane fusion by cryofixation is also limited by the lack of synchrony in most systems. The patch-clamp methodology (Neher and Marty, 1982) provides about 10-times higher resolution with regard to duration and size of fusion pore formation (Almers, 1990; Almers and Tse, 1990). From surface capacitance flickering it was postulated that an initially reversible 1 nm large pore is formed in the sub-millisecond time range by gap junction-like protein subunits, which rapidly dissociate and thus allow for lipid rearrangement and pore expansion (Almers, 1990). Freeze-fracture replicas showed ~10 nm large fusion spots (Knoll et al. 1991; for reviews, see Plattner, 1989, 1991), but in visualization of hydrophilic pores of ~1 nm the problem arises that they can be obscured by capillary condensation (Plattner and Zingsheim, 1983). No attempt has been made so far to exploit decoration effects (i.e. surface-specific heavy metal binding) or true replication with high-resolution shadowing methods (cf. Plattner and Zingsheim, 1983) to visualize pore formation and dissociation of subunits. In its most recent developmental stage cryofixation, however, allows for parallel biochemical analyses at defined time points after fusion triggering. To achieve this goal we have made the quenched-flow methodology applicable to intact, fragile cells, with a short dead time and a total “preparative” time resolution in the millisecond range (Knoll et al. 1991).

A correlation of data from freeze-fracture and patch-clamp analyses with biochemical data is now within reach and some interesting aspects are emerging. This also concerns the actual demonstration of oligomeric proteins or of protein aggregates in biomembranes capable of fusion (see below), as postulated from patch-clamp analysis (Almers, 1990).

Exocytosis

Stimulated exocytosis is the type of membrane fusion that has been analyzed most thoroughly. Cryofixation was applied to nerve terminals, adrenal glands, mast cells, egg cells, amoebocytes and Paramecium (protozoa) cells (for reviews, see Plattner, 1989, 1991). The smallest fusion pores recognizable are about 10 nm in size. Interpretation faces problems like coordination of freezing with exocytosis stimulation and discrimination of exocytosis from rapidly coupled endocytosis. All these aspects can now be overcome by quenched-flow analysis (though applicable only to cell suspensions). Yet so far only exocytosis in Paramecium cells has been analyzed under synchronous conditions by this method (Knoll et al. 1991); see Figs 1 and 2.

Common features emerging from the different methods include: (i) the long-known close association of IMPs and (ii) that of membrane-associated proteins with fusion sites in different systems (Plattner, 1981, 1991); (iii) the possible decay of IMPs into subunits during fusion, which can now be substantiated ultrastructurally at least in the Paramecium system (Knoll et al. 1991); see Fig. 2; and (iv) cryofixation and freeze-fracture analysis reveal the formation of pedestal-like depressions in the cell membrane during

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fusion in several systems (Chandler, 1988; Plattner, 1989). This might increase fusogenicity by high local curvature, resulting from high free energy in the bilayer (Blumenthal, 1987).

For exocytic membrane fusion in Paramecium, the time constant (for all events in a cell population) is 57 ms (Fig. 1). For the earliest fusion events recognizable (“focal fusion”, Fig. 2) a life-time of <1 ms can be extrapolated from its frequency during fast freezing under synchronous trigger conditions. (This estimation is possible by referring the number of focal fusion stages observed within a certain time to the total number of potential fusion sites analyzed during this time.) Although this approaches the resolution of the cryofixation method (Plattner and Bachmann, 1982), it agrees well with a similar estimation yet under different experimental conditions, i.e. during exocytosis inhibition by antibodies or F(ab)\textsubscript{2} fragments directed against the cell surface (Momayezi et al. 1987). Sub-millisecond values were obtained by patch-clamp analysis for other cell types (Almers, 1990; Almers and Tse, 1990). The coincidence of data from so widely different approaches is remarkable.

Paramecium is the system with the strictest structure-function correlation available so far. A comparison of exocytosis-competent and -incompetent strains shows that ultrastructurally clearly identifiable intrinsic and peripheral proteins are mandatory for fusion to occur (Beisson et al. 1980; Plattner et al. 1980; Pouphile et al. 1986; Plattner, 1991). With non-discharge mutants (containing secretory organelles docked at the cell membrane, but without the capability of exocytotic membrane fusion) these structural features can be induced, strictly in parallel to function repair, by cytosol transfer (Beisson et al. 1980; Lefort-Tran et al. 1981; Pape et al. 1988). So far 23 alleles from 13 genes are now known to affect fusion capacity (Bonnein et al. 1992), but the gene products remain to be elucidated. They might be required for fusion per se and/or for self-assembly of fusogenic proteins. Unfortunately it has not been possible to patch-clamp Paramecium cells in order to ascertain whether exocytotic membrane fusion would be paralleled by capacitance flickering as in other systems.

According to quenched-flow analysis with Paramecium cells, dephosphorylation of a soluble but structure-bound cortical protein occurs in parallel to membrane fusion (Höhne-Zell et al. 1992), i.e. within <80 ms under synchronous (95%) conditions (Knoll et al. 1991). The decay of IMP aggregates (“fusion rosettes”, proteins of unknown identity) into subunits (Fig. 2) would be fully compatible with the fusion concept derived from patch-clamp experiments in other cells (Almers, 1990). This rearrangement and/or perturbation of the fusing membrane might be initiated by dephosphorylation of the underlying phosphoprotein - a hypothesis still to be tested. Protein dephosphorylation is mandatory for exocytosis in other systems also (Churche et al. 1990; Whalley et al. 1991), although a time sequence analysis has not yet been made.

From patch-clamp analysis, pore formation by oligomeric proteins passing through both membranes to be fused has been postulated (Almers, 1990). Yet up to now no set of matching IMPs has been recognized in any of the exocytotic systems analyzed, possibly for reasons discussed below for viral systems.

At least in nerve terminals such intrinsic proteins would have to be pre-assembled to account for the low lag time and high fusion rate (Almers and Tse, 1990). Presumably, hexameric integral proteins occur not only in synaptic vesicles (synaptophysin; Thomas et al. 1988; Svp25: Volknandt

| Table 1. Comparison of data obtained (a) by cryofixation/freeze-fracturing and (b) by patch-clamp analysis |
|-------------------------------|------------------|------------------|
| **Fusion pore formation**     | Method (a)       | Method (b)       |
| Smallest size detectable     | <10 nm           | 1 nm             |
| Life-time (break-through)    |                  |                  |
| Exocytosis                   | 1 ms             | <1 ms            |
| Virus fusion                  | min              | min              |
| Detection of reversibility    | No               | Yes              |

**Rearrangement of components during fusion**

<table>
<thead>
<tr>
<th><strong>Protein subunit dispersal</strong></th>
<th>Membrane shape changes</th>
<th>Lipid transfer</th>
<th>Glycocalyx transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occasionally seen</td>
<td>Local protrusion</td>
<td>Not detectable</td>
<td>Detectable (ultra-thin sections)</td>
</tr>
<tr>
<td>Not detectable</td>
<td>Postulated</td>
<td></td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

**Fusion events to be analyzed**

<table>
<thead>
<tr>
<th><strong>Exocytosis</strong></th>
<th><strong>Endocytosis</strong></th>
<th><strong>Cell-cell fusion</strong></th>
<th><strong>Virus fusion</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes (applicable)</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

For details and references, see the text.
Fig. 2. Freeze-fracture aspects of the cell membrane during exo-endocytosis in *Paramecium*. Plasmatic (a–e) and exoplasmic (a'–e') fracture faces, shadowing from bottom to top. (a, a') Resting stage (no stimulation) with "rosette" (RO) IMP's assembled in the middle of a "ring" (RI) of IMP's which delineates the site of extrusion of secretory contents. (b, b') Focal fusion (arrowhead) on top of a pedestal-like depression of the cell membrane during exocytosis. While smaller IMP's (arrows) have been formed around the fusion zone, most rosette IMP's have disappeared, possibly by decay into monomers. One such event might suffice to initiate membrane fusion. (c, c') Expanded fusion pore whose diameter could increase to the size of a ring (not shown). (d, d') For endocytosis during retrieval of the organelle membrane we show for the first time focal fusion (arrowhead) quite similar to that during exocytosis. (e, e') After membrane resealing has been completed the small IMP's (arrow) persist in the center of a ring. Bar, 0.1 µm. (d, d') not published before; other micrographs are reproduced from Knoll et al. (1991) with permission of The Rockefeller University Press (New York).
et al. 1990), but also in the presynaptic membrane (physophilin: Thomas and Betz, 1990; mediatophore; Morel et al. 1991). A heterooligomeric complex of different intrinsic proteins in synaptic vesicle membranes has also been envisaged for fusion pore formation (Bennett et al. 1992). Crosslinking experiments showed the association of synaptotagmin with the latrotoxin receptor and from this their possible involvement in membrane fusion has been derived (Petrenko et al. 1991).

The wide variety of candidates for fusogenic proteins currently looks very intriguing. Hence, it is not yet possible to correlate strictly the biochemical data with their actual involvement in membrane fusion. Moreover, in different systems fusion would have to operate via different molecular constituents, since the candidates discussed are not ubiquitous.

Endocytosis

Less is known about membrane fusion during endocytosis. As indicated by the abrupt decay of surface capacitance in patch-clamp analyses (Almers, 1990), this is also a very fast process. For a Paramecium cell suspension, under synchronous conditions, the time constant is 126 ms for all events (Fig. 1). In this case, when endocytosis can be clearly dissociated from exocytosis on the ultrastructural level, freeze-fractures reliably show focal fusion, as we show for the first time (see Fig. 2d). From the relative frequency of endocytic focal fusion events a similar or slightly longer life-time than for exocytosis can be estimated, i.e, in the range of several milliseconds.

During endocytosis a small fraction of glycocalyx material becomes transferred to the organelle’s ghost membrane (Plattner et al. 1985). This is paralleled by transfer of lipids from the cell membrane to the ghost by pressure in the surface bilayer - a potential driving force for resealing (Monck et al. 1990). It remains to be explained (i) how the steric hindrance by the glycocalyx is overcome and (ii) which proteins would mediate fusion.

Cell-cell fusion

Freeze-fracture analysis of physiological cell-cell fusion was possible with myoblasts, but only after some synchronization (Knoll and Plattner, 1989). Fusion is focally restricted and not accompanied by IMP rearrangement. According to patch-clamp analysis virus-mediated cell-cell fusion is also of the focal type (Spruce et al. 1991). The same may hold for sea urchin egg-sperm fusion, since, according to patch-clamp analysis, it exceeds the time resolution (30 ms) available in recent experiments (McCulloh and Chambers, 1992).

In mammals a fusogenic protein (combined with a related subunit for cell-cell docking) in the sperm head reveals sequence homology with viral fusion proteins (Blobel et al. 1992). Considering the candidates discussed, e.g. for some exocytic systems (see above), one can no longer assume only one type of fusogenic protein for the different types of biomembrane fusion.

Membrane fusion via viral fusogenic proteins

Fusogenic proteins of some enveloped viruses, e.g. hemagglutinin (HA) of influenza virus, are well characterized (White et al. 1983; Stegmann et al. 1989; White, 1990). After binding to sialic acid (VanMeer and Simons, 1983), membrane fusion is accomplished by a conformational change exposing hydrophobic domains. However, the exact mechanism is not known and the following alternatives are discussed. (i) The hydrophilic domain of the fusogenic protein penetrates the target membrane (Stegmann et al. 1990); (ii) it penetrates the viral membrane (Ruigrok et al. 1986); or (iii) none of the membranes, but it forms a hydrophobic bridge between the membranes (Bentz et al. 1990).

No protein partner is necessary in the target membrane, since fusion can also be induced efficiently with liposomes (VanMeer and Simons, 1983). Freeze-fractures reveal focal fusion spots (Burger et al. 1987; Knoll et al. 1988) with specific IMPs interpreted as lipidic structures with long lifetimes. This corresponds to lipid fluorescence labeling assays (Knoll et al. 1988; Sarkar et al. 1989). Cell-cell fusion by the influenza HA-protein was analyzed by patch-clamp measurements, and a long-lasting fusion pore was observed (Spruce et al. 1989, 1991). Again a matching protein partner in the target membrane is not likely to be involved.

Intracellular membrane fusion processes

No comparative analyses could be done in these cases with patch-clamp and cryofixation methods. Only some occasional ultrastructural observations reveal focally restricted fusion (or budding?) areas (Fujikawa et al. 1990). However, there is increasing biochemical evidence of the occurrence of oligomeric protein aggregates in different endomembranes. Reports include membrane-associated proteins, e.g. of the NSF (NEM (N-ethyl-maleimide)-sensitive fusion protein) and SNAP (soluble NSF attachment protein) complex in the Golgi apparatus (Clary et al. 1990) and in endosomes (Diaz et al. 1989). Aspects to be addressed are their possible arrangement in subunits in association with some integral proteins (Wilson et al. 1992) and their actual involvement in docking and/or fusion processes.

Conclusions

There is an interesting merger of ultrastructural with electrophysiological data on formation, size and duration of biological membrane fusion. Biochemical analysis offers a variety of candidates for fusogenic, probably oligomeric, proteins. Different proteins are to be expected for the different systems. The actual involvement in membrane fusion now has to be ascertained (or dismissed) for each of them. Moreover, data from viral systems suggest that a set of matching gap junction-like proteins in both membranes to be fused may not always be required. Despite the considerable resolution of the patch-clamp technique, with results refining and improving on those obtained by cryofixation and freeze-fracturing, both methods have their own merits (see Table 1). Methods allowing for topological coordi-
tion will be required to localize fusogenic proteins to the site of actual membrane fusion.

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