Salient features of artificially induced cell fusion

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In early work done more than ten years ago in this laboratory, observations were made which indicated a role for osmotic swelling in chemically induced cell fusion (Akhong et al., 1973a). At that time it was suggested that: “As we have never observed fusion between the oval-appearing, unswollen erythrocytes, and as every chemical found to induce cell fusion in this laboratory also causes cells to become spherical, we conclude that cell swelling is an essential requirement in cell fusion” (Akhong et al., 1973b). More recently, Finkelstein and his colleagues have shown that phospholipid vesicles can be induced to fuse with a planar, phospholipid bilayer by osmotic forces. They have suggested that osmotic swelling of intracellular vesicles may be essential for the fusion of biological membranes in exocytosis (Akabas et al., 1984), and this is consistent with findings that solutions of high osmolarity can inhibit the exocytosis of chromaffin granules in adrenal medullary cells (Hampton & Holz, 1983; Pollard et al., 1984) and the exocytosis of cortical granules in sea-urchin eggs (Zimmerberg & Whitaker, 1985).

A mechanism for membrane fusion/fission processes

In this paper, a general scheme for the fusion of biological membranes is proposed. It is apparent that the first step in fusion/fission reactions is the provision of some means of bringing the two membranes very close together. This may involve Ca2+ ions, or proteins like synexin, or membrane receptors, but there are other ways of achieving the same end (e.g. diectrophoresis). Next, a change needs to occur that produces a “rupture site” in the membrane permeability or transport processes that results in osmotic swelling. In exocytosis, only the vesicle needs to swell, but in cell fusion the membranes of both cells would be affected. If excessive cell swelling occurs before the membranes of two cells fuse, the cells involved will lyse as individual cells and fusion will not occur. Under certain conditions, however, the molecules of the two bilayers that are being forced together may fuse into a single bilayer in small localized areas. (Three recent papers (Melikyan et al., 1983; Fisher & Parker, 1984; Horn, 1984) have supported an earlier proposal by Neher (1974) that a new bilayer is formed when two bilayers are pushed together). Perturbing agents, such as chemical fusogens or an electrical breakdown pulse, which decrease the lateral adhesion of phospholipid molecules, would be expected to facilitate the formation of such a single bilayer. Finally, continued osmotic swelling will cause membrane fusion to follow, in the case of cell fusion, by an osmotically driven rounding of the fused cells. Membrane fusion presumably occurs between the two cells because the rim at which the three bilayers meet is relatively weak. If the swelling continues, however, total lysis of the fused cells will also occur.

Membrane proteins and osmotically driven cell fusion

Following our early work we made numerous attempts to induce erythrocytes to fuse by osmotic means alone, but none of our experiments was successful. It has since become apparent that we were unsuccessful because mammalian and avian erythrocytes, unlike vesicles and bilayers of phospholipids, have a complex membrane skeleton of spectrin and actin that restricts shape changes. We have more recently shown that the chemically induced fusion of human erythrocytes involves the proteolysis of ankyrin by a serine proteinase, and proteolysis of band 3 by a Ca2+-activated cysteine proteinase (Quirk et al., 1978; Lang et al., 1984). Furthermore, inhibitors of these proteases inhibit fusion. At least three other groups of workers have also published data on the role of proteinases in chemically induced cell fusion (Kosower et al., 1983; Nakornchai et al., 1983; Thomas et al., 1983). The mechanism for fusion as outlined above, in which osmotic forces play a central role, must therefore be modified to allow for the influence of intrinsic membrane proteins, membrane skeletons, and cellular skeletons and also for the factors that modulate the influence of these structures. It should also be noted that a number of the agents that influence the fusion of biological membranes are multifunctional. For example, Ca2+ ions not only bring membranes into close contact, but also activate cysteine proteinases, and activate other enzymes, some of which, in turn, produce lipid fusogens such as acetylglucers and fatty acids.

My colleague, Dr Ahkong, and I have recently found that hen erythrocytes can be fused by an osmotic shock if proteolytic degradation of the restraining proteins has previously been initiated. Hen erythrocytes were attached to an Alcian-Blue-coated glass surface, treated with the ionophore A23187 (10 μg/ml) and 2 mM-Ca2+, and then incubated at pH 7.4 and 37°C for 2 h. After this long incubation, little if any fusion was apparent but, only 5 min after adding water to dilute the buffer from 150 mM to 60 mM, many binucleate cells were seen. In a related experiment some of the cells were labelled by pretreatment with membrane-permeable 6-carboxyfluorescein diacetate, which enters the cells and is enzymically hydrolysed to 6-carboxyfluorescein (Goodall & Alton, 1982). Only 5 min after the osmotic shock, fluorescent cells were fused with each other and also with non-fluorescent cells to produce weakly fluorescent, binucleate cells.

Poly(ethylene glycol)

As human erythrocytes are fused by the application of 40% poly(ethylene glycol) (PEG) of M, 6000 in the presence of 5 mM-EGTA, something other than Ca2+ ions must be responsible for the initial cell adhesion in this case. Some years ago we showed by differential scanning calorimetry that PEG, in concentrations that are sufficiently high to induce cell fusion (30–50%), has such a high avidity for water that no unbound water is present in these preparations of PEG (Blow et al., 1978). This withdrawal of water is considered to force membranes together but how PEG actually induces membrane fusion, rather than the mere close apposition of membranes, has previously not been apparent. However, like two other groups of workers (Kråding et al., 1978; Knutton 1979), we have never seen fusion in concentrated solutions of PEG. It is not until the PEG solution has been diluted with isotonic buffer, and the cells resuspended in an isotonic buffer, that cytoplasmic bridges between fused cells are observed. At this stage, the cells also swell and some of them lyse. Related experiments in my laboratory are consistent with this fusion reaction being driven by osmotic forces as a consequence of PEG making phospholipid membranes permeable to quite large molecules, although not permeable to

Abbreviation used: PEG, poly(ethylene glycol).
haemoglobin. Thus, treatment of phospholipid vesicles with fusogenic concentrations of PEG 6000 has been found to cause the release of entrapped arsenazo III (M, 776), and it even releases entrapped, radioactive PEG 4000. Also, fusogenic concentrations of PEG 6000 allow cells to fuse with erythrocytes within 1 or 2 min (Aldwinckle et al., 1982).

Monolayers of human erythrocytes, attached to Alcian-Blue-coated glass, and pre-labelled with carboxyfluorescein, can be fused by treatment with PEG. Entrapped carboxyfluorescein does not leak extensively from human erythrocytes in 40% PEG 6000, but, when the 40% PEG is diluted to 15% with buffer, the entrapped carboxyfluorescein immediately starts to leak from the cells thus preventing the fluorophore from being used as a marker for fusion. Lieber & Steck (1982) have shown that cations, especially Ca\(^{2+}\), decrease the size of the holes produced in human erythrocyte membranes by osmotic lysis, and we have similarly found that Ca\(^{2+}\) ions (50 \(\mu\)M) are particularly effective in decreasing the very rapid leakage of carboxyfluorescein (and the measured cell swelling) with erythrocytes that have been permeabilized by PEG. Under these conditions, it is possible to use the fluorophore as a cytoplasmic marker, and we have shown that fusion occurs when the concentrated solution of PEG is replaced, after dilution, by an isotonic buffer.

What happens to the membrane skeleton in cells treated with PEG? Surprisingly, there are at least two reports which indicate that PEG enters cells (Krähling et al., 1978; Szabó et al., 1982), and we have obtained supporting, independent evidence. The presence of PEG inside cells might be expected to precipitate the proteins of the membrane skeleton and this may facilitate cell fusion.

Electrofusion

Finally, I should like to turn to the fusion of erythrocytes that is obtained by applying an alternating field to align the cells by dielectrophoresis, and then subjecting them to a short high-voltage, direct current, pulse. (Here I wish to thank Professor U. Zimmermann and his colleagues for their generous help when we initially set up our electrofusion equipment.) The formation of pores in the membranes of erythrocytes, by a high-voltage pulse, causes colloid osmotic lysis (Zimmermann et al., 1976; Kinosita & Tsong, 1977). One might therefore expect the fusion of electro-permeabilized cells to be driven by osmotic lysis, especially since the breakdown pulse appears to damage the membrane skeleton (Donath & Arndt, 1984). We have again used carboxyfluorescein as a cytoplasmic marker to determine when fusion actually occurs and we have found that the transfer of fluorescence from labelled to unlabelled human erythrocytes can be virtually instantaneous with the electrical breakdown pulse. Thus we have observed the immediate formation of weakly fluorescent chains of four to seven cells by the fusion of one labelled cell with a number of unlabelled cells. During the course of our experiments, Sowers (1984) has published similar work using fluorescent isothiocyanate dextran, but he has fused erythrocyte ghosts rather than intact cells. Can colloid osmotic swelling occur so quickly that it can cause cell fusion to be virtually instantaneous with the voltage pulse? Zimmerberg et al. (1980) showed that swollen phospholipid vesicles fuse with a phospholipid bilayer under the influence of an applied osmotic gradient, but that more flaccid vesicles do not swell sufficiently under the same gradient for fusion to occur. In the fluorescent transfer experiments with electrically fused erythrocytes mentioned above, the cells were pre-swollen because they were suspended in a medium containing only 150 mM-erythritol. When less swollen cells (in a more concentrated medium containing 200 mM-erythritol) were used, the instantaneous fusion was much less and no chains of four or more fused cells were formed. This therefore indicates, as in Finkelstein's work with vesicles which were swollen to differing extents, that the electrically induced cell fusion is driven by osmotic forces. It is also interesting to note, however, that when the cells in 200 mM-erythritol subsequently swelled more extensively, before lysis, a delayed fusion occurred that was revealed by a later transfer of fluorescence.

In conclusion, I should like to point out that our observations on artificially induced cell fusion are relevant to the membrane fusion induced by those viruses that cause cell swelling and lysis, as well as to the biochemistry, biophysics and cell biology of exocytosis. It is also interesting to note, in relation to possible mechanisms for the release of neurotransmitters, that our experiments have indicated that osmotically driven membrane fusion can be an extremely rapid process.