Localized osmotic swelling and cell fusion in erythrocytes: possible implications for exocytosis

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Summary

Factors that govern the experimentally induced fusion of erythrocytes with one another may generally be relevant to whether or not osmotic forces drive membrane fusion in exocytosis because, under appropriate conditions, osmotic swelling can drive the fusion of erythrocytes. It is now reported that these cells fuse when they are subjected to osmotic swelling caused by exposure to small permeant molecules. The behaviour of erythrocytes in fusion induced by treatment with a concentrated solution of high molecular weight poly(ethylene glycol) (PEG) is also of specific interest in relation to exocytosis because the haemoglobin of erythrocytes that are dehydrated by concentrated solutions of the polymer may be regarded as a model for the tightly packed, dehydrated contents of the granules in secretory cells. We have observed that, under certain conditions of rehydration, the swelling of aqueous microdroplets between the dehydrated haemoglobin and the plasma membrane is closely associated with the fusion of partially rehydrated but still shrunken, PEG-treated erythrocytes. It is therefore apparent that osmotic forces, acting locally at the sites of aqueous microdroplets, can drive the fusion of membranes that encapsulate a dehydrated, concentrated protein, even though gross osmotic swelling at the level of the light microscope is absent. This finding is consistent with the possibility that osmotic swelling may play a role in exocytotic membrane fusion if it is restricted to a small zone immediately under the granule membrane.

Key words: osmotic swelling, erythrocytes, cell fusion, poly(ethylene glycol), exocytosis.

Introduction

Erythrocytes undergo cell fusion, as well as cell lysis, when they are swollen under specific conditions (Ahkong & Lucy, 1986; Lucy & Ahkong, 1988), and we have now found that they fuse on exposure to small permeant molecules. Since erythrocytes are permeabilized to Na⁺ and Ca²⁺ when they are dehydrated by concentrated solutions of high molecular weight poly(ethylene glycol) (PEG) (Blow et al. 1979), they also swell beyond their initial volume, lyse, and fuse when they are subsequently rehydrated. The latter fusion process occurs in two stages. Thus, continuity between the phospholipid bilayer membranes of adjacent erythrocytes (membrane fusion) is established in dehydrating solutions of PEG, but cytoplasmic continuity (membrane fission) does not occur until the cells are rehydrated. Membrane fission then takes place in 300–400 ms, which is consistent with the osmotic rupture of a membranous barrier between the permeabilized, swelling cells (Ahkong et al. 1987).

Many studies have indicated that osmotic forces are also important in a number of exocytotic systems. These include mucocyst secretion in Tetrahymena (Satir et al. 1973), the release of parathyroid hormone (Brown et al. 1978), endotoxin-induced degranulation in amoebocyte blood cells of the horseshoe crab, Limulus polyphemus (Ornberg & Reese, 1981), the discharge of nematocysts in sea anemones (Lubbock et al. 1981), exocytosis induced in the toad urinary bladder by antidiuretic hormone (Kachadorian et al. 1981), the release of insulin from the pancreas (Orci & Malaisse, 1980; Hermans & Henquin, 1986), the secretion of serotonin from platelets (Pollard et al. 1977), the cortical granule reaction in sea-urchin eggs (Zimmerberg et al. 1985; Zimmerberg & Whitaker, 1985), and the acrosome reaction in guinea-pig sperm (Green, 1982). However, in order for osmotic forces to be responsible for establishing continuity between the contents of secretory granules and the extracellular medium, as well as for subsequently expelling the contents of the granules (Green, 1982; Whitaker & Zimmerberg, 1987), osmotically driven changes must clearly precede the formation of the initial fusion pore. This point has been investigated in mast cells from beige mice by using changes in membrane capacitance to detect the moment
at which the granule membrane and the plasma membrane become continuous in exocytosis. In two laboratories, the change in capacitance was found to precede swelling of the granules, and it was concluded that osmotic swelling cannot be the driving force for membrane fusion, at least in this system (Zimmerberg et al. 1987; Breckenridge & Almers, 1987).

Recently, however, it has been commented that these observations on mast cells show only that membrane fusion precedes visible swelling, and that it is premature to conclude that swelling has no role to play in exocytotic membrane fusion since local swelling (restricted to a small zone immediately under the granule membrane) may still have a function in the fusion process (Green, 1987). It is relevant to this issue that we have consistently noted that, under certain rehydrating conditions, PEG-dehydrated erythrocytes fuse before they exhibit gross swelling. Fusion of the partially rehydrated, but still shrunken, cells has now been found to be associated with the swelling of aqueous microdroplets between the plasma membrane and the haemoglobin of the treated cells. Since the haemoglobin of PEG-dehydrated erythrocytes may be regarded as a model for the closely packed contents of secretory vesicles, this finding is consistent with the possibility that osmotic swelling may play a role in exocytotic membrane fusion if it is restricted to a small zone immediately under the granule membrane.

Materials and methods

6-Carboxyfluorescein diacetate was from Molecular Probes, Eugene, Ore., USA. PEG 300 and malonamide were from Sigma Chemical Company. PEG 6000 was from BDH, and it was purified as described by Smith et al. (1982).

Erythrocytes were washed and freed from leukocytes as described by Quirk et al. (1978). The cells were labelled with carboxyfluorescein, attached to Alcian Blue-coated coverslips, and investigated by fluorescence microscopy as described by Ahkong et al. (1987).

In Fig. 3A, 50 μl packed human erythrocytes were incubated for 5 min at 37°C with 50 % (w/v) PEG 6000 (1 ml) in a medium containing NaCl (110 mM), buffered with Heps (40 mM) at pH 7.4. The cells were centrifuged and resuspended in 35 % PEG 6000 for 10 min at 37°C, and subsequently fixed by the addition of an equal volume of 35 % PEG solution containing 2 % (v/v) glutaraldehyde. In Fig. 3B, human erythrocytes were incubated with 50 % PEG 6000 for 5 min at 37°C, and 10 volumes of the above saline solution (containing 0.5 %, v/v glutaraldehyde) was added. In Fig. 3C, human erythrocytes were treated with 40 % (w/v) PEG 6000 for 5 min at 37°C, and 10 volumes of buffered saline solution (containing 0.5 % glutaraldehyde) were added. All cells in Fig. 3 were post-fixed for 30 min in a solution of OsO4 (1 %, w/v) in cacodylate buffer (Quirk et al. 1978) containing Ruthenium Red (0.05 %, w/v), and stained for 2 h with a saturated solution of uranyl acetate (Vos et al. 1976).

Results

Osmotic swelling, lysis and cell fusion induced by permeant molecules

In previous work, human erythrocytes that had been incubated in an isotonic salt/buffer solution, which was progressively diluted and which contained 0.5 mM-La3+ to minimize lysis, were observed to fuse. Hen erythrocytes that had been pre-incubated with ionophore A23187 and 5 mM-Ca2+ to cause a proteolytic breakdown of the membrane skeleton, also fused when an osmotic shock was applied (Ahkong & Lucy, 1986).

The susceptibility of erythrocytes to osmotically induced cell fusion is now further demonstrated by the finding that fusion, as well as lysis, occurs when human erythrocytes are exposed to permeant molecules such as malonamide (Whittam, 1964) and the small polymer, PEG 300. This observation is of particular interest in the present context because it is comparable to that made in model exocytosis studies on the osmotically induced fusion of stachyose-containing phospholipid vesicles with a planar phospholipid bilayer following entry of glucose into the vesicles (Cohen et al. 1982). When human erythrocytes are exposed to non-ionic solutions of either malonamide or PEG 300, they exhibit colloid osmotic swelling and lysis, which is inhibited by the presence of impermeable molecules, e.g. raffinose, sucrose or serum albumin. Calcium ions are known to stimulate a decrease in the size of holes produced by osmotic lysis in human erythrocyte membranes (Lieber & Steck, 1982), and Ca2+ also facilitates the fusion of erythrocytes under a variety of conditions. In our experiments, 10 mM-Ca2+ decreased osmotic lysis and facilitated the fusion of erythrocytes when the cells were incubated with 600 mM-malonamide or 500 mM-PEG 300 (15 %, w/v) (Fig. 1A). Like the lysis which occurred in the absence of Ca++, cell swelling and cell fusion were almost completely inhibited by the presence of serum albumin (60 μg ml−1) (Fig. 1B), and this further supports the interpretation of the fusion process in terms of osmotic forces.

Osmotic swelling, lysis and cell fusion induced by high molecular weight PEG

Paradoxically, the fusion of erythrocytes induced by concentrated, dehydrating solutions of high molecular weight PEG also depends on osmotic swelling. Thus, although the dehydrating action of 40 % PEG 6000 induces a continuity between adjacent erythrocyte membranes, which allows fluorescent probes to diffuse from labelled to unlabelled membranes (Wojcieszyn et al. 1983; Ahkong et al. 1987), cytoplasmic continuity between the fusion erythrocytes is not established until the cells, which are permeabilized to external cations by dehydrating concentrations of PEG (Aldwinckle et al. 1982; Blower et al. 1979), swell beyond their initial volume on rehydration in an isotonic medium. Lysis and cell fusion then both occur (Ahkong et al. 1987). In dehydrating solutions of PEG, erythrocyte haemoglobin may be virtually insoluble since fluorescent serum albumin in the cytoplasm (introduced by hypotonic lysis) is known to be immobilized when the cells are suspended in a 44 % PEG of high molecular weight (Wojcieszyn et al. 1983). Since the dry weight content of secretory vesicles tends to be some 50 % or more, which is indicative of a very high degree of condensation, an additional interest of the osmotically driven fusion of PEG-dehydrated erythro-
Fig. 2. Fluorescent micrographs of a monolayer of human erythrocytes in which 10% of the cells were cytoplasmically labelled with carboxyfluorescein. A. Cells incubated for 5 min at 37°C in a solution of PEG 6000 (50% w/v), containing NaCl (110 mM) and Hepes buffer (40 mM) at pH 7.4, showing uniform, partially quenched fluorescence. B. The same cells, 30 s after the 50% PEG was replaced by 35% PEG, showing bright, fluorescent microdroplets and crescents in the cytoplasm. C. Another field, 5 min later. The developing fluorescence of previously unlabelled cells (arrowhead) shows that they have fused with adjacent, cytoplasmically labelled, fluorescent cells. ×630.
cytes therefore lies in some similarity of the physical state of their haemoglobin to that of the closely packed contents of such vesicles. Many of the secretory granules in human pulmonary mast cells contain crystalline structures (Caulfield et al. 1980), the contents of mucocysts in *Tetrahymena* are also crystalline (Satir et al. 1973), and insulin is stored in the secretory vesicles of pancreatic B cells of most species as an insoluble aggregate with a crystalline structure (Gold & Grodsky, 1984).

When human erythrocytes are cytoplasmically labelled with carboxyfluorescein and treated with a 40% solution of purified PEG 6000, the cells remain uniformly fluorescent as the polymer solution is subsequently diluted to 13% and then replaced by an isotonic buffer (Ahkong et al. 1987). In contrast, labelled erythrocytes that are more extensively dehydrated in 50% PEG 6000 (Fig. 2A) develop a second cytoplasmic phase when the polymer is replaced by 35% PEG. This is indicated by the striking, immediate formation of brightly fluorescent microdroplets (50–700 nm diameter) and crescents, many of which are closely adjacent to the plasma membrane (Fig. 2B). Electron micrographs show that haemoglobin is absent from the new phase (Fig. 3), and we therefore attribute its formation to water entering the cells more rapidly than it is absorbed by the immobilized haemoglobin. A considerable swelling pressure apparently develops in the aqueous droplets since a localized ballooning of the plasma membrane is seen when the droplets lie between the membrane and the haemoglobin (Fig. 3A). The enhanced fluorescence of the new phase is presumably due to the absence of fluorescence quenching of the carboxyfluorescein by haemoglobin.

Surprisingly, cell fusion occurs between these partially rehydrated but still shrunken cells, as is demonstrated by the movement of cytoplasmic carboxyfluorescein from labelled to unlabelled erythrocytes (Fig. 2C). Electron micrographs reveal, however, that microdroplets, lying between the contorted plasma membranes of the dehydrated cells and their haemoglobin, are closely associated with the majority of the fusion sites (Fig. 3B–C). It thus appears that the swelling of aqueous microdroplets that are confined between the haemoglobin and the plasma membrane, rather than gross cell swelling, provides the...
osmotic driving force for the fusion of these shrunken erythrocytes.

Microdroplets form throughout the cytoplasm of both human and hen erythrocytes when the cells are dehydrated in 50% PEG 6000, which is then diluted to 25%-40% with isotonic buffer. On further addition of isotonic buffer, the aqueous microdroplets are absorbed into the rehydrating haemoglobin, and the cytoplasm becomes uniformly fluorescent again. Microdroplets are also produced when erythrocytes are dehydrated with 40% PEG and are then exposed to 5% PEG (Fig. 3C), also produced when erythrocytes are dehydrated with 25%-40% with isotonic buffer. On further addition of osmotic driving force for the fusion of these shrunken cells are subjected to a larger decrease in osmolarity.

Discussion

Whether or not a shared bilayer functions as an intermediate structure during cell fusion (Lucy & Ahkong, 1986), the electron micrographs of PEG-dehydrated erythrocytes that contain aqueous microdroplets indicate that the membrane-fission event in the fusion of these cells is driven by highly localized osmotic swelling. As far as experimentally induced cell fusion is concerned, this is probably an isolated phenomenon, which arises from the use of specific conditions for rehydrating the erythrocytes, which results in entering water forming transient, but discrete, aqueous microdroplets before it is absorbed by the haemoglobin. In contrast, the fusion of erythrocytes exposed to small permeant molecules (Fig. 1A), to lipid-soluble fusogens (Lucy & Ahkong, 1988), and to dehydrating concentrations of high molecular weight PEG used as previously described (Ahkong et al. 1987) is associated with gross cell swelling. The present observations nevertheless strongly indicate that localized osmotic forces, acting at the sites of microdroplets of water that are immediately adjacent to membranes, can be responsible for the fusion of membranes that encapsulate dehydrated proteins even though gross osmotic swelling, at the level of the light microscope, is demonstrably absent.

Interestingly, in a rapid-freezing study of exocytosis in amoebocyte blood cells, the earliest change seen was a separation between the granule core and its membrane, which left a clear crescent beneath the membrane of the storage granule. It was considered that this could have been due to an influx of water resulting from an osmotic gradient (Ornberg & Reese, 1981).

Although caution clearly needs to be exercised in extrapolating from cell fusion phenomena in erythrocytes to the exocytosis of secretory granules, the several observations reported here are consistent with the proposal that osmotic swelling may play a role in exocytotic membrane fusion if it is restricted to a small zone immediately under the granule membrane (Green, 1987).

In the light of our observations, we suggest that consideration should be given to the possibility that a short, but finite, delay between the entry of water into secretory granules and its absorption by their immobilized contents could provide the basis for osmotically driven, membrane fusion–fission phenomena in exocytosis. Such a delay could be due to one or more factors, including the relatively slow dissolution of granule contents that occurs in some examples of exocytosis, inhomogeneous swelling of gel-like granule contents, and an involvement of proteolytic events in the dissolution process.

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References


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