Changes in the Distribution of Intramembranous Particles in Hen Erythrocytes during Cell Fusion Induced by the Bivalent-Cation Ionophore A23187

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Incubation of hen erythrocytes with Ca^{2+} and the bivalent-cation ionophore A23187 induced slight cell fusion in 1 h at 37°C, and extensive fusion during a subsequent 15 min at 47°C. Redistributions of intramembranous particles were observed, possibly involving interactions between Ca^{2+} and phospholipids, which are discussed in relation to molecular mechanisms of cell fusion.

This paper is concerned with relationships between membrane ultrastructure and function during chemically induced cell fusion; our observations are relevant to the biochemistry of membrane fusion and the behaviour of membrane proteins in many cellular functions, including secretion by exocytosis, in *Tetrahymena* (Satir *et al.*, 1973), in the posterior pituitary (Dempsey *et al.*, 1973) and in mast cells (Lawson *et al.*, 1976).

The bivalent-cation ionophore A23187 promotes the fusion of hen erythrocytes in the presence of exogenous Ca^{2+} (Ahkong *et al.*, 1975b). Since ionophore A23187 allows Ca^{2+} to accumulate in rat and human erythrocytes (Reed & Lardy, 1972; Kirkpatrick *et al.*, 1975), it has been suggested that an increased cytoplasmic Ca^{2+} in hen erythrocytes treated with the ionophore may cause intramembranous particles to aggregate and, perhaps more importantly for cell fusion, give rise to protein-free regions of lipid bilayer (Ahkong *et al.*, 1975a,b). Ultrastructural observations are now reported which provide support for these concepts.

Materials and Methods

Suspensions of neuraminidase-treated fresh hen erythrocytes (approx. 2×10^8 cells/ml) in a buffered salt solution (pH 7.4) (Ahkong *et al.*, 1975b), containing dextran (80 mg/ml) (mol.wt. 82000) from Sigma (London) Chemical Co. (London S.W.6, U.K.), were incubated with Ca^{2+} (2 mM) and ionophore A23187 (6.6 µg/ml) at 37°C and 47°C. Ionophore A23187 was given by Eli Lilly (Windlesham, Surrey, U.K.). The preparation of the hen erythro-

cytes was as previously described (Ahkong *et al.*, 1973, 1975b). The ionophore was dissolved in ethanol (1 mg/ml). In control experiments, neuraminidase-treated cells were incubated either in the above medium with ethanol (6.6 µl/ml) in the absence of ionophore but in the presence of Ca^{2+} (2 mM) or with ionophore in the presence of Ca^{2+} (2 mM) and EGTA [ethanedioxybis(ethylamine)tetra-acetate] (4 mM).

Small portions of cell suspension were taken for light-microscopy at intervals during the incubations. After 1 h at 37°C or after a further incubation at 47°C for 15 min, samples (1 vol.) for electron microscopy were fixed at 0°C for 1 h in a solution (2 vol.) of glutaraldehyde (2%, v/v) in sodium cacodylate/HCl buffer (0.1 M), pH 7.4 (Glauert, 1975). For freeze-etching, the fixed samples were incubated in approx. 8 ml of glycerol (30%, v/v) at room temperature (approx. 20°C) for 2 h and subsequently frozen in chlorodifluoromethane (Freon-22), half-fluid:half-solid, at the temperature of liquid N_2 . The frozen samples were fractured at -100°C, heat-etched at the same temperature for 1 min and immediately replicated in a Balzers BAF 301 high-vacuum freeze-etch unit by using the single specimen stage. Samples for thin-sectioning were post-fixed for 30 min in a solution of OsO_4 (1%, w/v) in the cacodylate buffer containing Ruthenium Red from TAAB Laboratories (Reading, Berks., U.K.), (0.05%, w/v) (Pate & Ordal, 1967), stained for 2 h with a saturated aqueous solution of uranyl acetate, dehydrated through an acetone series (70%, 90%, 100%), embedded in Araldite epoxy resin from CIBA (ARL) Ltd. (Duxford, Cambridge, U.K.) and sectioned with an LKB Ultratome III. Replicas and thin sections were mounted on untreated 400-mesh copper grids and examined in an AEI EM 6B electron microscope at 60 kV.

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Results

On incubation at 37°C in medium containing Ca^{2+} and dextran, neuraminidase-treated hen erythrocytes aggregate into large clumps in which individual cells are not easily distinguished (Ahkong *et al.*, 1975b). In the additional presence of the bivalent-cation ionophore A23187, small vesicles bud from these cells during the first 15 min of incubation. A similar membrane budding has been reported (Allan *et al.*, 1976) and also observed in this laboratory for human erythrocytes treated with ionophore A23187. Freeze-fractured preparations of vesicles released from hen erythrocytes were found to be free from intramembranous particles (Plate 1d) by comparison with the remaining plasma membranes of treated cells (Plate 1a) and with the membranes of cells from a control experiment (Plate 1c).

Most of the cells were rounded and swollen after 1 h at 37°C with ionophore and Ca^{2+} . The nuclei were then more easily seen (Plate 2a), but thin sections showed that only about 1% of the total cell population was involved in cell fusion. Interestingly, however, freeze-fracturing of these preparations revealed that numerous roughly circular areas (0.1–0.2 μm in diameter), which were almost completely free from intramembranous particles, were randomly distributed over the P fracture face of the cells (Plate 1a) [for the nomenclature of the faces see Branton *et al.* (1975)]. Elsewhere, the intramembranous particles in these cells were unusually closely packed (Plate 1a) as compared with cells from a control experiment (Plate 1c). Since the membranes were fixed with glutaraldehyde before freezing, the appearance of Plate (1a) is unlikely to be an artifact of freezing or of treatment with glycerol (McIntyre *et al.*, 1974).

On transferring the cells to 47°C for 15 min, extensive cell fusion occurred that was easily seen by light-microscopy (Plate 2b); electron microscopy indicated that more than 80% of the cells were fused (Plate 2c). Freeze-fractured preparations of the fused cells showed that a noticeable change had occurred in the distribution of the intramembranous particles of the P fracture face. The particles were clustered in random aggregates, with disruption of the previously bare circular areas (Plate 1b).

Numerous vesicles (0.1–0.8 μm in diameter) were present at the periphery of the multinucleated cells (Plate 2c). These vesicles, which were seldom observed in the cells at 37°C, carried intramembranous particles. As judged by the relative location of the E and P fracture faces, the vesicles are apparently inside-out with respect to the orientation of the plasma membrane (Plate 1e), and therefore seem to be isolated remnants of plasma membrane resulting from multiple adjacent sites of membrane fusion between neighbouring hen erythrocytes.

In the absence of ionophore, or in the presence of ionophore with Ca^{2+} and EGTA, no morphological changes of any kind were observed in the cell clumps, and the P fracture face (Plate 1c) resembled that of untreated cells.

In similar experiments with human erythrocytes that were treated with ionophore A32187 and Ca^{2+} , little if any movement of intramembranous particles was observed at 37°C; further, despite the membrane budding at 37°C mentioned above, no cell-to-cell fusion was seen at either 37°C or 47°C.

Discussion

Poste & Allison (1973) have suggested that the aggregation of intramembranous protein particles is important in membrane fusion. This is supported by observations in several systems, namely secretion in *Tetrahymena* (Satir *et al.*, 1973), the secretion of vasopressin (Dempsey *et al.*, 1973), the degranulation of mast cells (Lawson *et al.*, 1976), the fusion of Golgi-derived vesicles (Gratzl & Dahl, 1976) and the fusion of hen erythrocytes by Sendai virus (Bächi *et al.*, 1973). Also, dimethyl sulphoxide and glycerol, which cause aggregation of intramembranous particles in lymphocytes (McIntyre *et al.*, 1974), induce hen erythrocytes to fuse (Ahkong *et al.*, 1975a). The present paper provides a direct demonstration of the movement of intramembranous particles in chemically induced cell fusion.

Whether or not membrane fusion is mediated by the interdigitation of membrane proteins, as additionally proposed by Poste & Allison (1973), or by the intermingling of membrane lipids in protein-free areas of lipid bilayer after protein aggregation (Ahkong *et al.*, 1975a) has not yet been established, although circumstantial evidence appears to favour fusion in the lipid regions of membranes (Satir *et al.*, 1973; Dempsey *et al.*, 1973; Lawson *et al.*, 1976). Ca^{2+} may interact with spectrin molecules on the cytoplasmic surface of the plasma membrane to aggregate integral membrane proteins and, as pointed out previously (Ahkong *et al.*, 1975b), this may occur in cell fusion induced by an increase in the concentration of cytoplasmic Ca^{2+} . However, it is noteworthy that the binding of Ca^{2+} to phosphatidylserine in a bilayer yields solid aggregates of phosphatidylserine and allows other phospholipids present to form a separate fluid phase (Ohnishi & Ito, 1974; Jacobson & Papahadjopoulos, 1975). Should this occur in the cytoplasmic half of the plasma membrane (P fracture face), where most of the phosphatidylserine is located in erythrocytes (Zwaal *et al.*, 1973), the aggregated intramembranous proteins would probably be restricted to the fluid phase (cf. Grant & McConnell, 1974). Circular regions of bare lipid bilayer in Plate 1(a), which are free from intramembranous particles, may thus represent solid aggregates of phospholipid arising

EXPLANATION OF PLATE I

Distribution of intramembranous particles during cell fusion induced by the bivalent-cation ionophore A23187 and Ca^{2+}

Neuraminidase-treated hen erythrocytes were incubated at 37°C for 1 h (a and d), and at 47°C for a subsequent 15 min (b, c and e), either with Ca^{2+} (2 mM) and ionophore A23187 (6.6 μg/ml) (a, b, d and e) or with Ca^{2+} (2 mM) and ethanol (6.6 μl/ml) (c). The incubation medium used is described in the Materials and Methods section. Vesicles (v) released into the medium (med), from the cells incubated at 37°C with Ca^{2+} and ionophore A23187, were particle-free (d). Numerous roughly circular areas, free from intramembranous particles, were present only on the P fracture face of cells incubated at 37°C with Ca^{2+} and ionophore A23187 (a). The P fracture face of cells incubated at 37°C and subsequently at 47°C showed disruption of the previously bare circular areas (b). Intracellular vesicles in these preparations (e) were inside-out with respect to the orientation of their plasma-membrane fracture faces. A fractured convex inner half of a vesicle is seen to have a distribution of intramembranous particles that is known to be characteristic of the outer half of the plasma membrane (E); fractured inner half of plasma membrane (P); cytoplasm (cyt).

EXPLANATION OF PLATE 2

Cell fusion induced by the bivalent-cation ionophore A23187 and Ca^{2+} , as observed by light-microscopy (a and b) and electron microscopy (c)

Neuraminidase-treated hen erythrocytes were incubated at 37°C for 1 h (a), and at 47°C for a subsequent 15 min (b and c), with Ca^{2+} (2mM) and ionophore A23187 (6.6 μg/ml). The incubation medium used is described in the Materials and Methods section. Swelling of the clumped cells occurred at 37°C (a); extensive cell fusion within these clumps was observed at 47°C (b and c). The nuclei and numerous newly formed vesicles are preferentially located at the periphery of a multi-nucleated cell (c). For (a) and (b) magnification is $\times 1000$.

where exogenous Ca^{2+} has penetrated to the cytoplasm. [A comparable exclusion of proteins from solidifying regions of lipid on cooling of *Acholeplasma* and bacterial membranes has been observed by James & Branton (1973) and Haest *et al.* (1974).] Raising the temperature to 47°C increases lipid fluidity; the bare regions then no longer exclude intramembranous proteins (Plate 1b). Such an interpretation may imply that cell fusion at 47°C involves the intermixing of pools of fluid bilayer lipids in adjacent cells rather than the interdigitation of protein particles, since the latter are already free to move at 37°C when only about 1% of the cells fuse.

Absence of cell fusion in experiments with human erythrocytes and ionophore A23187 was consistent with the observed lack of movement of intramembranous particles in the treated cells, and with the well-documented immobility of these particles in fresh 'ghosts' from human erythrocytes (Elgsaeter *et al.*, 1976). The formation of membranous vesicles that nevertheless pinch off from both human and hen erythrocytes treated with ionophore A23187 at 37°C need not necessarily involve the movement of individual particles, as the overall contraction of an intact meshwork of spectrin and actin in fresh 'ghosts' from human erythrocytes compresses the lipid bilayer, causing it to 'bleb off' particle-free protein-free vesicles (Elgsaeter *et al.*, 1976). Vesicles produced from hen erythrocytes treated with ionophore A23187 are likewise free from intramembranous particles (Plate 1d), and those obtained similarly from human erythrocytes are markedly deficient in protein (Allan *et al.*, 1976). Thus these findings appear also to be consistent with the idea that membrane fusion proceeds after the emergence of protein-free areas of lipid bilayer (Ahkong *et al.*, 1975a).

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