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Membrane-mobility agent-promoted fusion of erythrocytes: Fusibility is correlated with attack by calcium-activated cytoplasmic proteases on membrane proteins

(fusion mechanism/membrane protein motion/fusion-potent lipid areas)

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ABSTRACT **Rat, but not human, erythrocytes undergo fusion promoted by the membrane-mobility agent 2-(2-methoxyethoxy)-ethyl *cis*-8-(2-octylcyclopropyl)octanoate (A_2C). The difference in behavior is correlated with rat erythrocyte membrane protein degradation caused by Ca^{2+}-activated proteases. The human erythrocyte is deficient in such protease activity. Membrane protein degradation is a necessary, but not sufficient, requirement for membrane fusion. Membrane protein degradation probably releases membrane components from certain constraints. In addition, the motion of membrane components precedes fusion and must be promoted by reagents such as A_2C, leading to the creation of fusion-potent lipid areas. This sequence of chemical and physical events occurs in other fusion processes.**

Membrane fusion is a ubiquitous cellular process, mediating such phenomena as fertilization, exocytosis, endocytosis, phagocytosis, and turnover of intracellular organelles (1, 2). Fusion can be induced experimentally by certain viruses (3) and chemical agents (4, 5). Membrane-mobility agents are long-chain esters designed to promote motion of molecules in cell membranes. Membrane-mobility agents, which enter cells via the small particles formed on dispersion in aqueous medium, promote the lateral mobility of certain ligand–membrane receptor complexes and inhibit cytokinesis in some types of cells (5–9). Membrane-mobility agents, especially 2-(2-methoxyethoxy)ethyl *cis*-8-(2-octylcyclopropyl)octanoate (A_2C), are efficient promoters of cell fusion; the results obtained using a defined agent such as A_2C have allowed the identification of stages in the overall fusion process and the formulation of a molecular mechanism for membrane fusion (10–13).

We have previously found differences in the occurrence of A_2C-induced fusion among various mammalian non-nucleated erythrocytes as well as among avian nucleated erythrocytes (12, 13): erythrocytes of some species fuse easily, whereas those of others do not. "Mixed fusion," that between fusible erythrocytes from different species, is promoted by A_2C. Moreover, the potential for fusion is a transferable characteristic, fusion being induced by A_2C in otherwise nonfusible cells if mixed with fusible erythrocytes (13). The molecular basis for the differences observed among the erythrocytes of various species and for the transfer of fusibility has not yet been clarified.

We now show that the difference between fusible rat erythrocytes and the nonfusible human cells is correlated with proteolytic activity; degradation of rat cell membrane proteins is caused by a Ca^{2+}-activated cytoplasmic protease(s). The human erythrocyte is deficient in this protease activity. Membrane protein degradation is shown to be a necessary, but not sufficient, prerequisite for membrane fusion. Release of membrane components from constraints imposed by the cytoskeleton, allowing membrane component mobility, is proposed as one of the steps in the fusion process. Furthermore, the mobility of the membrane components must be promoted by a reagent such as the membrane-mobility agent A_2C, leading to the creation of fusion-potent lipid areas.

MATERIALS AND METHODS

Erythrocytes. Heparinized blood from rats (Charles River-derived albino female rats) and from healthy humans was centrifuged, the plasma and buffy coat were removed, and the erythrocytes were washed three times with 150 mM NaCl, with repeated removal of the upper layer of the cells. In some experiments, erythrocyte suspensions were freed from leukocytes and platelets by passing the suspensions through a column of microcrystalline cellulose-α-cellulose as described by Beutler *et al.* (14). The erythrocytes were then washed in 75 mM sodium acetate/70 mM NaCl, pH 5.6 [buffer A, a buffer previously used for erythrocyte fusion (11)].

Erythrocyte suspensions (8% packed cells) in buffer A were mixed with Ca^{2+} (final concentration, 0.1–1.0 mM) and the ionophore A23187 (final concentration, 10–20 μM, using a stock solution of 2 mM in acetonitrile), referred to as Ca/Iph. Samples were incubated at 37°C for 10–60 min. After incubation, 2 mM EDTA was added to all samples, and the cell suspensions were placed on ice. Ghosts were prepared from the cells and solubilized by established methods (15, 16).

Rat Erythrocyte Ghosts and Hemolysate. Because rat hemoglobin tends to precipitate at pH 6–8, certain modifications of established methods were developed for the preparation of ghosts and of hemolysate. Ghosts were prepared from NaCl-washed erythrocytes by hemolysis in 5 mM phosphate buffer (pH 8.2), followed by washing with 20–30 vol of 10 mM NaCl to remove hemoglobin. Ghosts were suspended in 150 mM NaCl. Membrane-free hemolysate was prepared from cells washed in buffer A by the addition of 2 vol of 5 mM sodium acetate (pH 5.6) to 1 vol of cells. After a few minutes, sufficient 3 M NaCl was added to make the suspension isotonic, and then it was centrifuged at 43,000 × *g* for 30 min to remove membranes and precipitated hemoglobin. All procedures were carried out at 4°C.

Ghost suspensions were mixed with Ca^{2+} with or without the addition of hemolysate (final concentrations; ghost suspension containing 0.5–0.8 mg of protein per ml, 5 mM Ca^{2+}, hemo-

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Abbreviations: A_2C, 2-(2-methoxyethoxy)ethyl *cis*-8-(2-octylcyclopropyl)octanoate; Ca/Iph, calcium–ionophore combination.

lysate with 0.6–0.8 mM hemoglobin). Ghost suspensions were kept for 15 min on ice, and then incubated at 37°C for 30–60 min. This procedure led to trapping of hemolysate within sealed ghosts in the presence of Ca^{2+}. Aliquots were also incubated in the presence of 1 mM leupeptin or with 10 mM histamine. After incubation, 5 mM EDTA was added to the ghost suspensions, followed by centrifugation at 4°C, removal of supernatant, and solubilization.

Fusion of Erythrocytes and of Ghosts. Rat erythrocytes were suspended at a hematocrit of 8% in buffer A containing dextran (100 mg/ml) (T-70, Pharmacia) and 1 mM Ca^{2+}, as described (11, 13), or 1 mM EDTA. Ghosts, resealed as described above, were suspended in buffer A containing dextran (40 mg/ml). Cell or ghost suspensions were mixed with an equal volume of A_2C suspension [prepared by sonication of 0.5 μl of A_2C per ml of 150 mM NaCl for about 30 sec (11)]. Suspensions were incubated at 37°C and mixed gently every 5–10 min. Aliquots were removed at intervals for observation by light- or phase-contrast microscopy and for photography. After an appropriate time, EDTA was added (final concentration, 2–5 mM); the suspensions were centrifuged at 4°C, converted to ghosts, and centrifuged; solubilized samples were then prepared (15, 16).

Analysis of Membrane Proteins. Analysis was carried out by electrophoresis on $NaDodSO_4$/acrylamide slab gels, followed by fixation, staining, and photography by established methods (16, 17).

RESULTS

Calcium-Induced Changes in the Intact Rat and Human Erythrocyte. Rat and human erythrocytes were incubated in buffer A (pH 5.6) either with or without Ca/Iph. The membrane proteins were analyzed by gel electrophoresis (Fig. 1). Membrane protein degradation occurred in rat erythrocytes that had been treated with Ca/Iph (500 μM/10 μM) for 20 min. The spectrin bands, band 2.1, and band 3 diminished markedly with the appearance of multiple bands of lower molecular weight in the regions between bands 2.1 and 3 and below band 3. Very little, if any, high M_r protein was present. Incubation of rat cells with 0.05–0.1 mM Ca^{2+} for 10 min was sufficient to cause significant protein degradation (data not shown). No changes were observed in the protein patterns from cells incubated with EDTA, with ionophore alone, or with calcium alone (data not shown).

In contrast, very significant amounts of high M_r proteins were found in membranes isolated from human erythrocytes treated with Ca/Iph under the same conditions (Fig. 1). As Lorand *et al.* (18) have shown, high M_r proteins are produced by such treatment of human erythrocytes arising from the action of a Ca^{2+}-activated cytoplasmic transglutaminase (18, 19).

Calcium-Induced Changes in the Rat Erythrocyte Ghosts. Gel electrophoretic patterns of membrane proteins from rat erythrocyte ghosts incubated with Ca^{2+}, with or without hemolysate, are shown in Fig. 2. The protein patterns of ghosts incubated with Ca^{2+} alone ("white" ghosts) were only slightly different from those of ghosts incubated with EDTA, with partial formation of a new band of slightly lower molecular weight in the band 3 area (Fig. 2, lane B vs. lane A). However, when rat hemolysate was present in the ghosts ("red" ghosts), incubation with Ca^{2+} led to marked changes in the membrane proteins, the patterns showing a very marked decrease in band 3, some diminution in band 2.1, and a slight diminution in the spectrin bands. In addition, high M_r proteins were observed (lane C). To a great extent, leupeptin inhibited the alterations in band 3 without inhibiting the formation of high M_r proteins and even somewhat enhancing their formation in the leupeptin-treated ghosts (lane D). Histamine, on the other hand, inhibited the formation of high M_r proteins but did not prevent the alterations in bands 3 and 2.1 (lane E).

Fusion of Rat Erythrocytes and of Ghosts and Alterations in Membrane Proteins. The fusion of rat erythrocytes was induced by A_2C, as described (13) (Fig. 3*A*). Fusion was inhibited by EDTA (Fig. 3*B*). Rat white ghosts loaded with Ca^{2+} could not be fused by A_2C (Fig. 4*A*). In contrast, extensive fusion of

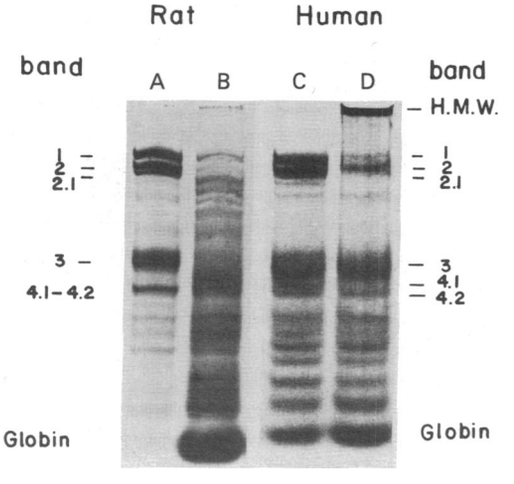

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoretic patterns of erythrocyte membrane proteins isolated from rat and from human cells treated with Ca^{2+} and ionophore. (Controls: no additions.) Cells (8% packed) were suspended in buffer A and treated with 0.5 mM Ca^{2+}/10 μM ionophore, or with ionophore alone, at 37°C for 20 min (rat cells) or 90 min (human cells). Cell suspensions were then mixed with 2 mM EDTA, cooled to 4°C, and centrifuged, and ghosts were prepared and solubilized. H.M.W., high M_r. Lanes: A and C, controls; B and D, with Ca^{2+}.

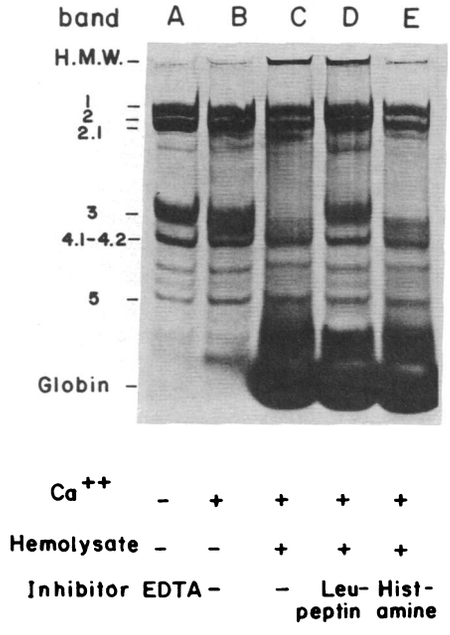

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoretic patterns of proteins isolated from rat erythrocyte ghosts. Ghost suspensions were mixed with the following: lane A, EDTA (5 mM); lane B, Ca^{2+} (5 mM); lane C, Ca^{2+} (5 mM) and hemolysate (0.8 mM hemoglobin); lane D, Ca^{2+}, hemolysate, and leupeptin (1 mM); lane E, Ca^{2+}, hemolysate, and histamine (10 mM). Suspensions were incubated at 37°C for 60 min, mixed with 5 mM EDTA, cooled, centrifuged, and solubilized. H.M.W., high M_r.

red ghosts (i.e., ghosts loaded with Ca^{2+} and cytoplasmic proteins) was induced by A_2C (Fig. 4*B*). No fusion occurred in red ghosts in the absence of A_2C (Fig. 4*C*). Leupeptin inhibited fusion of A_2C-treated red ghosts (10–20% fusion vs. 70–80% in the control), whereas histamine did not inhibit fusion (data not shown).

The gel electrophoresis profiles of membrane proteins derived from fusing cells or ghosts are shown in Fig. 5. The protein pattern for A_2C-treated EDTA-treated nonfusing cells serves as a control, the profile being shown in Fig. 5 (lane A). Alterations were observed in the profiles for intact fusing rat cells. Some bands diminish, especially in the 1, 2, 2.1, and 3 regions, and lower molecular weight bands appear, accompanied by little high M_r protein (lane B). Similar marked alterations in protein patterns were found for A_2C-treated fusing red ghosts (lane E), except for a greater increase of high M_r proteins. These alterations were similar to those observed in nonfusing red ghosts (lane D). Profiles of membrane proteins of white ghosts treated with or without A_2C showed only slight changes in the region of band 3 (lanes C and F).

DISCUSSION

The membrane-mobility agent A_2C promotes fusion of rat erythrocytes, but not of human erythrocytes (13). We now show that fusibility depends on a calcium-activated protease and that both membrane protein degradation and membrane component mobility are necessary for fusion to occur.

The response of the rat erythrocyte membrane to Ca^{2+} (entry promoted by A23187 ionophore) (Ca/Iph) differs from that of the human erythrocyte. After exposure to Ca/Iph, significant rat erythrocyte membrane protein degradation occurs, accompanied by only limited amounts of high M_r proteins, whereas little degradation of human erythrocyte membrane proteins is seen along with significant amounts of high M_r proteins. The Ca^{2+}-induced rat erythrocyte membrane protein degradation is dependent on cytoplasmic factor(s): Ca^{2+} has little effect on white ghosts (i.e., ghosts devoid of cytoplasm), whereas marked membrane protein alterations (much degradation and little high M_r protein formation) do occur in red ghosts (i.e., in the presence of hemolysate).

A_2C-promoted fusion of cells or ghosts requires calcium and is inhibited by EDTA. It is accompanied by protein degradation similar to that observed in the rat cell loaded with Ca/Iph. There is thus a strong suggestion that A_2C promotes the entry of calcium into the cell, because no ionophore is used in the A_2C-promoted fusion. This conclusion is strengthened by the finding that A_2C, which is incorporated into the human erythrocyte membrane (20), promotes significant high M_r protein formation in human erythrocyte membranes in calcium-containing medium (unpublished results).

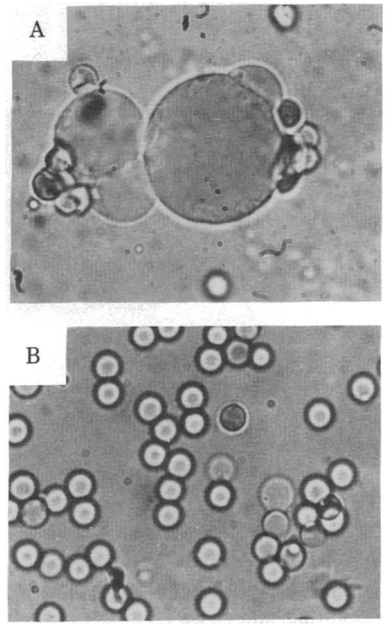

FIG. 3. Micrographs showing A_2C-induced fusion in rat erythrocytes. Cells were suspended in buffer A containing Ca^{2+} or EDTA, mixed with A_2C, then incubated at 37°C for 45 min. (*A*) Ca^{2+} (0.5 mM). (*B*) EDTA (1 mM).

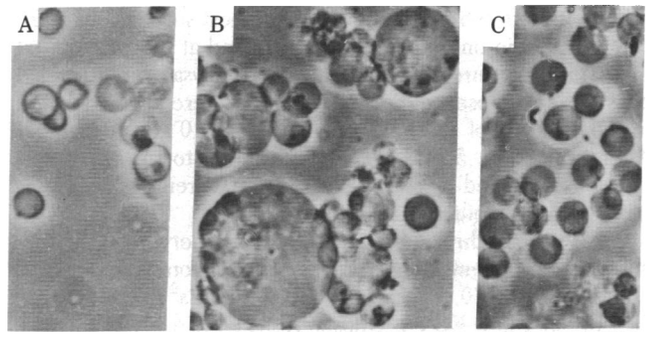

FIG. 4. Micrographs showing A_2C-induced fusion of rat erythrocyte ghosts. Ghosts were resealed in the presence of Ca^{2+} and hemolysate (red ghosts) or with Ca^{2+} and dextran at 100 mg/ml (white ghosts) (43), centrifuged, and resuspended in buffer A, containing dextran at 40 mg/ml. Ghost suspensions were then mixed with an A_2C suspension in 150 mM NaCl or with NaCl alone and incubated at 37°C for 45 min. (*A*) White ghosts treated with A_2C. (*B*) Red ghosts treated with A_2C. (*C*) Red ghosts incubated without A_2C.

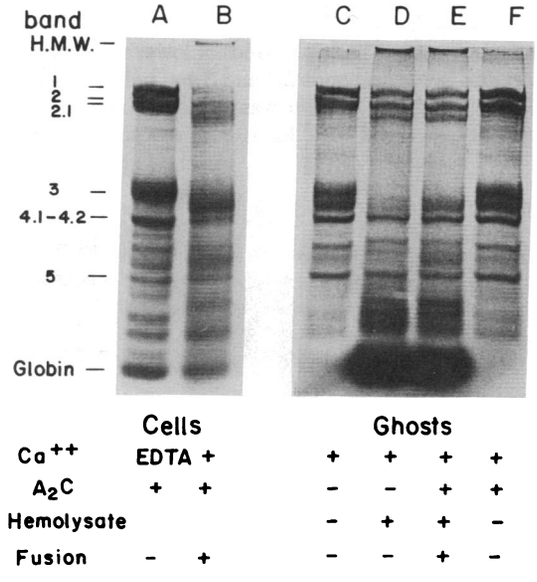

FIG. 5. $NaDodSO_4$/polyacrylamide gel electrophoretic patterns of membrane proteins derived from fusing and nonfusing rat cells and ghosts. Lanes A and B, erythrocytes. Aliquots of cell suspensions used for the fusion experiment described in Fig. 3 legend were mixed with 2 mM EDTA; ghosts were prepared and processed for gel electrophoresis. Lane A, cells treated with EDTA (1 mM); lane B, cells treated with Ca^{2+} (0.5 mM). Lanes C–F, ghosts. Aliquots of ghost suspensions used for the fusion experiment described in Fig. 4 legend were mixed with 5 mM EDTA, centrifuged, and solubilized. Lane C, white ghosts treated with NaCl; lane D, red ghosts treated with NaCl; lane E, red ghosts treated with A_2C; lane F, white ghosts treated with A_2C. H.M.W., high M_r.

Rat erythrocyte cytoplasm does contain both Ca^{2+}-activated protease(s) and transglutaminase. In red ghosts, protein degradation but not high M_r protein formation, is inhibited by leupeptin. High M_r protein formation, but not protein degradation, is suppressed by histamine, a competitive inhibitor of transglutaminase (18, 19). Limited high M_r protein formation in the intact rat erythrocyte membrane reflects either less transglutaminase than protease activity, or that high M_r proteins are formed but are susceptible to protease.

In contrast, significant high M_r protein formation occurs under these conditions in human erythrocyte membranes, promoted by a cytoplasmic, Ca^{2+}-activated transglutaminase (18, 19). Protein degradation in human erythrocyte membrane has also been reported, mostly for ghosts incubated for many hours (21–25). Various Ca^{2+}-dependent and Ca^{2+}-independent proteases have been identified and isolated from human erythrocytes (26–29). Degradation of transmembrane proteins, band 3 and glycophorin, as shown by immunochemical methods, occurs in the Ca^{2+}-loaded, intact human erythrocyte in addition to formation of high M_r proteins in these cells (30). Glycophorin degradation appeared to be more extensive than that of band 3 (30). Under our conditions, extensive protein degradation occurs in the Ca^{2+}-loaded rat cell on a short incubation.

The facts may be summarized as follows: (*i*) Fusion requires calcium; (*ii*) calcium leads to membrane protein degradation; (*iii*) protease-induced membrane protein degradation must accompany fusion; (*iv*) membrane-mobility agent promotes membrane component motion; (*v*) membrane-mobility agent is required for fusion; and (*vi*) motion of membrane components is therefore required for fusion.

The present study shows that membrane protein degradation is necessary but not sufficient for the membranes (of cells or ghosts) to undergo fusion. Membrane protein motion must also precede fusion. Thus, the membrane-mobility agent A_2C leads to fusion of Ca^{2+}-treated red ghosts (membrane protein degradation occurs), but not of Ca^{2+}-treated white ghosts (no membrane protein degradation). In the absence of A_2C, fusion does not occur in intact rat erythrocytes, Ca^{2+}-loaded via ionophore A23187, or in red ghosts (both cases: membrane protein degradation occurs).

Protease activity may be necessary for a variety of natural and experimental membrane fusion events (1, 31–34). Ca^{2+}-activated proteases are found in a variety of cells (35–37), but the possible connection of any of these to membrane fusion has not been previously established. The protease(s) necessary for fusion could be activated directly or indirectly (31) (i.e., via another enzyme) by calcium. Calcium seems to be a requirement for most types of fusion; it has been suggested that the function of fusion-promoting agents is to introduce calcium into the cell (33). The actual role of protease activity in the overall fusion process has not been clarified (31, 33, 34). We show here that the introduction of calcium *per se* and the activation of protease activity do not suffice for the induction of fusion.

It is generally agreed that membrane fusion occurs in protein-free lipid areas and that membrane proteins have to move and redistribute to create such lipid, fusion-potent membrane regions. The motion of some membrane proteins is restrained by cytoskeletal elements. In the case of erythrocytes, the intrinsic membrane protein, band 3, is bound to the major cytoskeletal protein, spectrin, via ankyrin (band 2.1) (38–41). Spectrin deficiency facilitates fusion (42), whereas antispectrin antibodies inhibit fusion (43). Thus, partial degradation of the cytoskeleton, of the binding proteins, or of intrinsic proteins could release the restraints. Allowing the membrane proteins to move is not enough, and mobility must be enhanced with a reagent such as A_2C.

The present results are incorporated into an expanded scheme (Scheme I) for membrane fusion, especially as promoted by A_2C.

A_2C particle + cell
↓ sticking via hydrophobic bonding
A_2C particle–cell contact complex
↓ particle–membrane local fusion via inverted micelle mechanism
A_2C particle–cell fusion complex
↓ A_2C lateral diffusion throughout cell membrane
Cell with A_2C-loaded membrane
↓ 1. A_2C-promoted Ca^{2+} entry
2. Ca^{2+}-activated protease
Cell with degraded membrane protein
↓ A_2C-promoted membrane component motion
Cell with fusion-potent areas (FP-cell)
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FP-cell + FP-cell
↓ sticking
Cell–cell contact complex
↓ membrane–membrane fusion via inverted micelle mechanism
Cell–cell fusion complex
↓ porous barrier disappearance via "vesicles"
Fused cell

Scheme I

A detailed molecular model for the role of the inverted micelles in fusion has been presented previously (11). The general outlines of the mechanism proposed for A_2C-promoted membrane fusion probably apply to many other types of fusion, such as that induced by Sendai virus (3, 43–46), and they appear to be valid for at least some types of natural fusion processes.

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